Critical Study on the Development and Design of an Automated Multicapillary Electrophoresis Instrument with Collection of Mutant DNA Fractions Using Constant Denaturant Capillary Electrophoresis (CDCE)

by

Leslie E. Kao

SUBMITTED TO THE DEPARTMENT OF MECHANICAL ENGINEERING IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

BACHELOR OF SCIENCE IN ENGINEERING
AT THE
MASSACHUSETTS INSTITUTE OF TECHNOLOGY

February 2008

Signature of Author: _____
Department of Mechanical Engineering
December 12, 2007

Certified by: _____
William G. Thilly
Professor of Toxicology
Thesis Supervisor

Accepted by: _____
John H. Lienhard V
Professor of Mechanical Engineering
Chairman, Undergraduate Thesis Committee

Critical Study on the Development and Design of an Automated Multicapillary Electrophoresis Instrument with Collection of Mutant DNA Fractions Using Constant Denaturant Capillary Electrophoresis (CDCE)

by

Leslie E. Kao

Submitted to the Department of Mechanical Engineering
On December 12, 2007 in partial fulfillment of the
requirements for the Degree of Bachelor of Science in Engineering
as recommended by the Department of Mechanical Engineering

ABSTRACT

Constant Denaturant Capillary Electrophoresis (CDCE) is a separation tool based on the cooperative melting equilibrium principle that is used to detect mutations as low as of 10^{-6}. This technique has already demonstrated invaluable clinical applications in correlated preventative prognosis, medical evaluations, and interventions. Accordingly, there is a high demand to utilize CDCE as a cost-effective, high-throughput screening and separation technique to detect mutations in large DNA pooled samples.

The aim of this thesis is twofold: to describe DNA separation theories and technologies, as well as CDCE separation theory and applications; and to describe and analyze the design of and modifications applied to an integrated automated multicapillary instrument with collection of mutant fractions by using CDCE to meet the stringent requirements for detecting low-frequency mutations in pooled samples from large populations. The modified SCE2410 24-capillary DNA Sequencer, HTMS Model (High-throughput Mutational Spectrometer) by Q. Li *et al.* has been identified as the instrument that best meets these requirements.

This thesis will analyze this integrated HTMS instrumental design and modifications involving the multicapillary cartridge, the optical detection device, six independently controlled solid-state thermal heaters for the thermostat array in the CDCE temperature control system, and automated matrix replacement and fraction collection. The overall HTMS system design has led to results of high optical sensitivity (1×10^{-12}M fluorescence in detection limits), precise and stable temperature control ($\pm$ 0.01°C), and automated sample delivery, injection, matrix replacement, and fraction collection.

Thesis Supervisor: William G. Thilly
Title: Professor of Biological Engineering

ACKNOWLEDGEMENTS

First and foremost, I am deeply grateful to my thesis advisor, Dr. William G. Thilly, for whom I have the utmost respect and appreciation for all he has done for me.

I would also like to extend my gratitude to my Mechanical Engineering Academic Advisors, Dr. Alexander H. Slocum.

I am also grateful to the members of the Thilly Lab, who were always patient and helpful to me, providing assistance when I needed it and answering my questions. Thanks also go to former and current colleagues and/or students of Dr. Thilly, Per O. Ekstrom, Elena Gostjeva, Konstantin Khrapko, Xiao Li-Suchelelki, Weiming Zheng, and Shuo Zhang to name a few, for providing enormous research information on my thesis topic and lending me their analytical skills to help direct my research. Reference to their publications in this thesis are the best testament of their accomplishment. Grateful appreciation also goes to Rita Demeo, whose encouragement and assistance went beyond the call of duty at MIT.

Finally, to my parents, Drs. George and Cassandra Kao, for their unconditional love and support.

Grateful acknowledgments are made to Wiley-VCH Verlag GmbH & Co. KG for permission to use the following figures in this thesis: Figure 2, 4, 6, 7. 8, 9, 10, 11, 12, 13, 14, and 15.

LIST OF ABBREVIATIONS

A	adenine
ACDCE	automated denaturant capillary electrophoresis
AS-PCR	allele specific polymerase chain reaction
bp	base pair(s)
BRCA	breast cancer gene
C	cytosine
CAE	capillary array electrophoresis
CDCE	constant denaturant capillary electrophoresis
CDGE	constant denaturant gel electrophoresis
cDNA	complementary DNA
CE	capillary electrophoresis
CTCE	cycling temperature capillary electrophoresis
DCE	denaturant capillary electrophoresis
DGGE	denaturing gradient gel electrophoresis
dHPLC	denaturant high performance liquid chromatography
DNA	Deoxyribonucleic Acid
dsDNA	double stranded DNA
G	guanine
GC-clamp	guanine and cytosine rich domain
KRAS	Kirsten RAS gene, Ki-RAS
LIF	laser-induced fluorescence
MAMA	mismatch amplification mutation assay
Mb	mega bases
mRNA	messenger Ribonucleic Acid
PCR	Polymerase Chain Reaction
SNP	Single Nucleotide Polymorphism
SSCA	single strand confirmation analysis
SSCP	single strand conformation polymorphism
ssDNA	single stranded DNA
T	thymidine
TGCE	temperature gradient capillary electrophoresis
TGGE	temperature gradient gel electrophoresis
Tms	temperature of 50% melting equilibrium
TP 53	Tumor protein p53
TTGE	temporal temperature gel electrophoresis

LIST OF FIGURES

Table of Contents

Chapter 1

Introduction

1.1. Purpose of the Study

Mutation detection is a vital tool for increasing scientific understanding in the area of molecular biology and genetics [1]. The *Human Genome Project,* launched in 1990, with the goal of understanding the genetic make-up of the human genome sequence and increasing the availability of genetic sequence data, has expanded and enhanced the scope of discovery of the causes of mutations, the diagnosis and identification of genetic diseases, and the screening of populations for specific mutations [2]. Two significant areas have been emphasized by the academic and clinical research communities: First, feasible and reliable studies of correlated preventative prognosis, medical intervention and/or treatment, and evaluation of therapeutic outcomes have become a medical reality. Second, the demand for advanced methods and technology that can be utilized for high-throughput screening and separation techniques to detect mutations in large DNA pooled samples and for identifying and isolating point mutations has increased in urgency.

Point mutations are mutations where a small number (such as a single base pair) of base pairs are deleted, inserted, or substituted by a different base. A single-point mutation with an allele frequency of 1% or greater is known as a single nucleotide polymorphism (SNP), while point mutations occurring at allele frequencies of less than 1% are widely referred to as low-frequency mutations, or simply "mutations" [3-4].

However, there are some practical problematic considerations related to these methods for detecting low-frequency mutations in a large number of genetic and population studies, such as the screening of populations carrying inherited genetic risks for specific diseases. Namely, these considerations relate to testing and labor costs, as well as issues of reliability [3-5]. In order to address these problems, the aim of this thesis is firstly to provide a theoretical and methodological study related to DNA and the Constant Denaturant Capillary Electrophoresis (hereafter CDCE) separation techniques, and the use of the CDCE method to identify and isolate point mutations in pooled samples with high-speed and high-throughput DNA diagnoses and detection of mutation in 100-bp sequence with a sensitivity at least as low as 10^{-6} [7-11]. Secondly, to provide a critical assessment of the technological development, characteristics, and design of an automated multicapillary electrophoresis instrument with the collection of mutant DNA fractions by using CDCE as a cost-effective and reliable DNA separation technique [4-6]. Based on CDCE separation techniques, and coupled with fluorescence detection and a multicapillary cartridge, the selected model in this analysis herein is a substantially modified and commercially available SCE2410 24-capillary sequencer DNA instrument (SpectruMedix, State College, PA), HTMS Model (High-throughput Mutational Spectrometer Model), by Q. Li *et al.* [4].

1.2. Rationale for Instrumentation Needs

Capillary electrophoresis (CE) is a well-established method for the electrophoretic separation of a wide variety of biopolymers such as DNA, proteins, and polysaccharides

[13-14]. In recent years, CE has been applied more frequently to the detection of DNA polymorphisms and mutations in genetics. A sub-category, the constant denaturant capillary electrophoresis (CDCE), is based on a cooperative melting equilibrium principle [16-17] and was first developed on a laboratory-assembled instrument by MIT and Northeastern University groups in 1994 [6]. This technique is an invaluable tool for mutation detection with high sensitivity and low-frequency measurement [7-9].

CDCE is based on the same separation principles that underlie both denaturant gradient gel electrophoresis (DGGE) [16-17] and constant denaturant gel electrophoresis (CDGE) [22] with capillary electrophoreses (CE) of DNA in linear polyacrylamide metrics, introduced by Cohen *et al.* in 1988 [23]. Khrapko *et al.* explained that CDCE separation occurs in the heated area of the capillary, where faster-moving and unmelted DNA fragments are in equilibrium with slower-moving and partially-melted forms. Within a certain temperature range, the position of the melting equilibrium and the average electrophoretic mobility of each mutant are different. Due to these mobility differences, sequences containing single base pair point mutations are separated from each other, and are able to identify each of the multiple peaks of a separation profile [6].

In comparison, among the various mutation detection methods – from direct sequencing to the allele-specific polymerase chain reaction (AS-PCR) method – CDCE is the most suitable technology because of its capability to: reduce the time it takes to separate mutants, increase separation efficiency, detect low-frequency mutations in human cells and tissues with mutant fractions as low as 10^{-6} [7], and identify mutants with a precision similar to that of direct sequencing [9].

1.3. Brief Review of Previous Studies Conducted Using CDCE

In a quick review of CDCE application with respect to the detection of various low-level frequency mutations and other unique features, including: (1) early research by Kumar *et al.* (1995) who demonstrated that combining high-fidelity PCR and CDCE to separate wild-type and mutant N-*ras* exon 1 and 2 sequences [25]. (2) Researchers, such as in MIT, Northeastern University, or Radium, utilized CDCE method for the analysis of DNA variation of several fragments, namely mitochondrial *DNA APC, HRAS, KRAS* exon 1, and *TP53* exon 8 [15, 10, 29, 38]. And (3) with further improvement of CDCE by modifying a commercially available instrument, in 2000, Ekstrom *et al.* reported using two-point fluorescence detections coupled with automated fraction collection to detect samples with mutant fractions as low as 10^{-4} in a single capillary instrument [28].

It is evident that CDCE analysis is well suited for use with a single capillary instrument to detect extremely low-frequency mutations in human cells and tissues, and can be adapted for use with any commercial DNA sequencing instrument for CDCE, as suggested by Khrapko *et al.* earlier [9]. However, in the multicapillary formats—for example, when applied to mutation detection in large populations—CDCE is limited by flexibility, automation, high-throughput, and reproducibility issues [4]. Accordingly, new improvements were added or recommended to these single- or multicapillary instruments. For instance:

(1) Ekstrom *et al.* reported in 2002 that blood from 4,000 individuals was pooled, and single-samples were analyzed by temperature gradient capillary electrophoresis (TGCE)

using a standard multicapillary DNA sequencing instrument MegaBACE™ 1000 DNA Analysis System (Amersham Pharmacia Biotech, Oslo, Norway) to compensate for temperature differences within the analysis chamber and between the capillaries [33].

(2) Bjorheim *et al.* in 2001 states that automated constant denaturant capillary electrophoresis was applied to detect the *KRAS* exon 1 mutation, heated in combination with a chemical denaturant to screen for *KRAS* mutations in an unmodified single capillary instrument, ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) [34].

(3) The same group also reported in 2002 that 191 *KRAS* samples were analyzed on the same commercial instrument, MegaBACE™ 1000 DNA Analysis System (Molecular Dynamics, Sunnyvale, CA), with only one minor modification -- the installation of a 96-capillary solid-state thermostat to allow the instrument to reach a high and precisely controlled separation temperature [35]. Accordingly, it provided the opportunity for the high-sensitivity screening of a large cancer population for *KRAS* exon 1 mutations [35].

(4) Based on comparative analysis, Minarik *et al.* recommended to adapting cycling temperature gradient method in commercially available instruments in order to achieve accurate temperature control and reproducibility [37]. As demonstrated by Kristen *et al.* in 2002 [38], or Bjorheim *et al.* in 2003 [44], that a 96-capillary instrument was adapted to cycling temperature capillary electrophoresis (CTCE) for direct identification of all oncogenic *KRAS* mutations in exon 1 without the need for sequencing.

(5) Recently, in 2005, Li-Sucholeiki *et al.* conducted a study of the detection and frequency estimation of rare variants underlying the inheritance of risk for common disease from large populations proved that CDCE-based mutational spectrometry of DNA pools could indeed offer a feasible and cost-effective means of testing [20].

Besides cost and efficiency, in a multicapillary instrument for mutation detection of less than 0.1%, high sensitivity and dynamic range are required for CDCE application [4]. However, commercially-available multicapillary DNA sequencers provide relatively narrow dynamic range and compromised sensitivity compared to single capillary systems, which are optimized for measurements in a narrower spectral range [4]. Consequently, improved optical detection by multicapillary instruments is recommended for mutation detection [4, 7, 10].

An additional area that requires improvement, as suggested by Q. Li *et al.,* is the collection of mutant DNA fractions, or fraction collection [4]. In general, commercial multicapillary DNA sequencers are not equipped with automated fraction collection capabilities. An improvement in automated fraction collection systems with enrichment of the mutant fractions will increase the speed and scale of its capability for low frequency mutation detection [4, 7, 10-11].

1.4. Goals and Chapter Descriptions

The goals for this thesis are twofold: the first part focuses upon DNA separation theories and technologies, as well as CDCE separation theory and applications; the second part concentrates on the description and analysis of an improved, automated multicapillary electrophoresis instrument with fraction collection.

Applying a force to this flexure system will cause it to deflect as shown in the bottom right section of Figure 4.

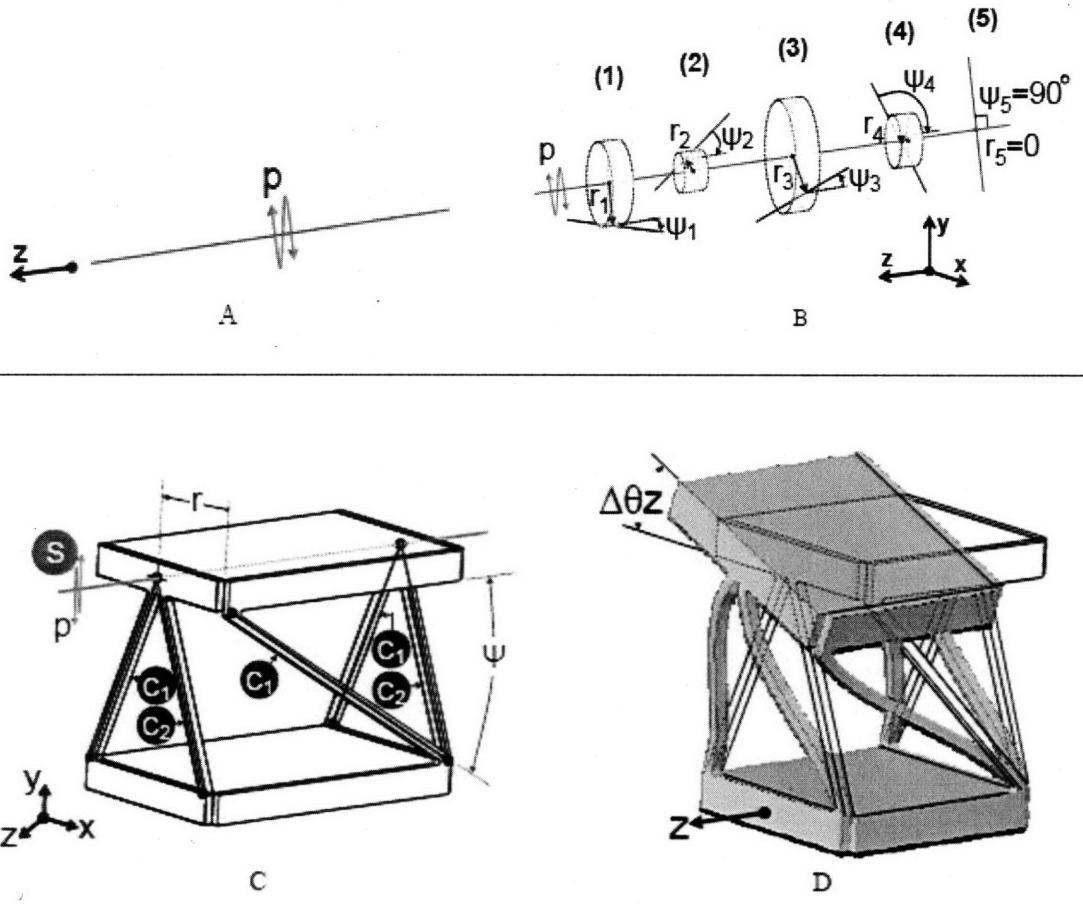


Figure 4: Topology of constraint and freedom sets of a screw flexure and its physical geometry and expected motion [2].

A more detailed mathematical foundation for screw theory and the mathematics behidn flexures with a single screw degree of freedom may be found in the Hopkins and Culpepper paper on FACT [2].

The remaining chapters 6 and 7 will cover the reconstructed experimental results and discussion on this high-throughput mutational spectrometer's major modules, including fraction collection, in conjunction with an overall evaluation of standard detection sensitivity, dynamic range and crosswalk, CDCE temperature optimization, separation reproducibility, and the result of a 12-fold enrichment of the mutant after fraction collection. As reported by Q. Li *et al.*, the aforementioned capabilities have demonstrated the potential to provide HTMS process detection limit of $10^{-4.5}$ for this instrument [4]. This potential will make the detection of 1 mutant out of 20,000 alleles in pooled blood samples a feasible reality [3-4], which will consequently reduce the overall cost of mutation detection and benefit future large-scale SNP and mutation analysis studies [4, 27].

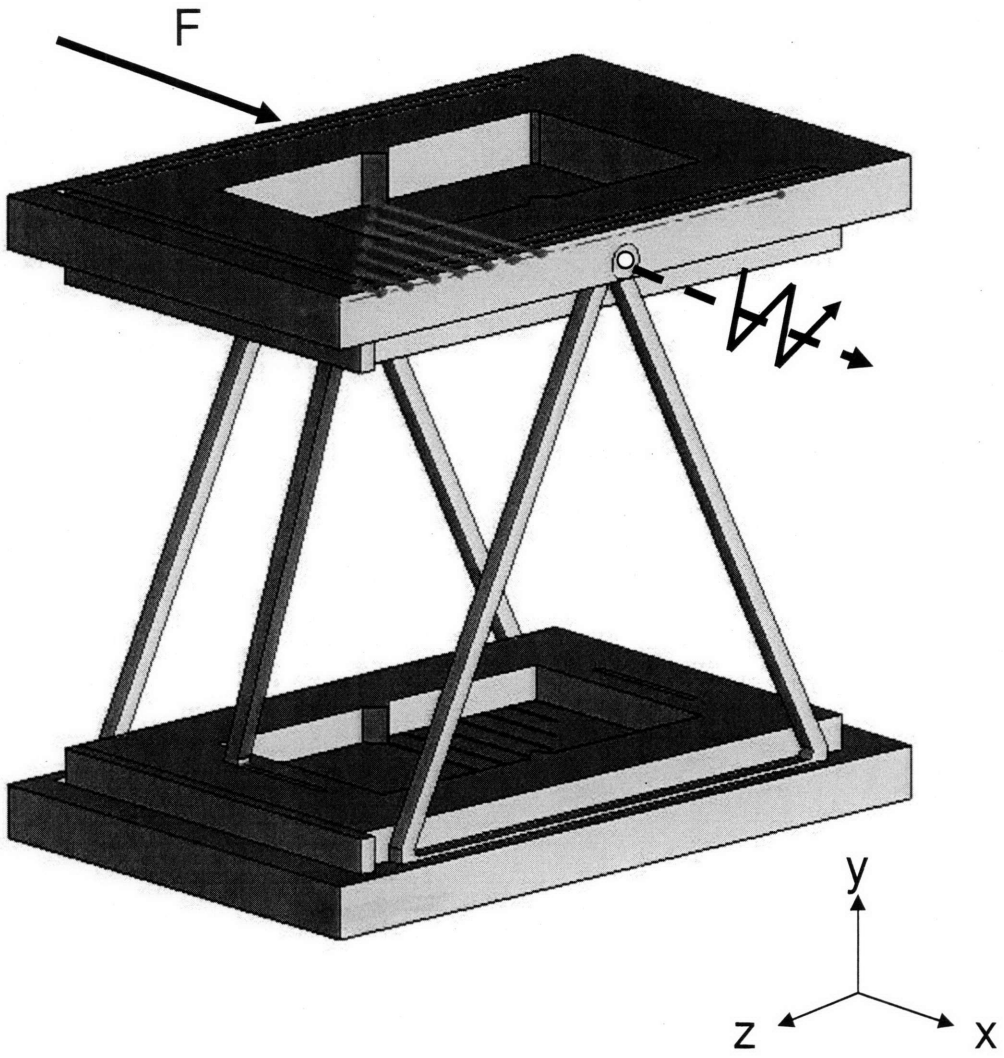


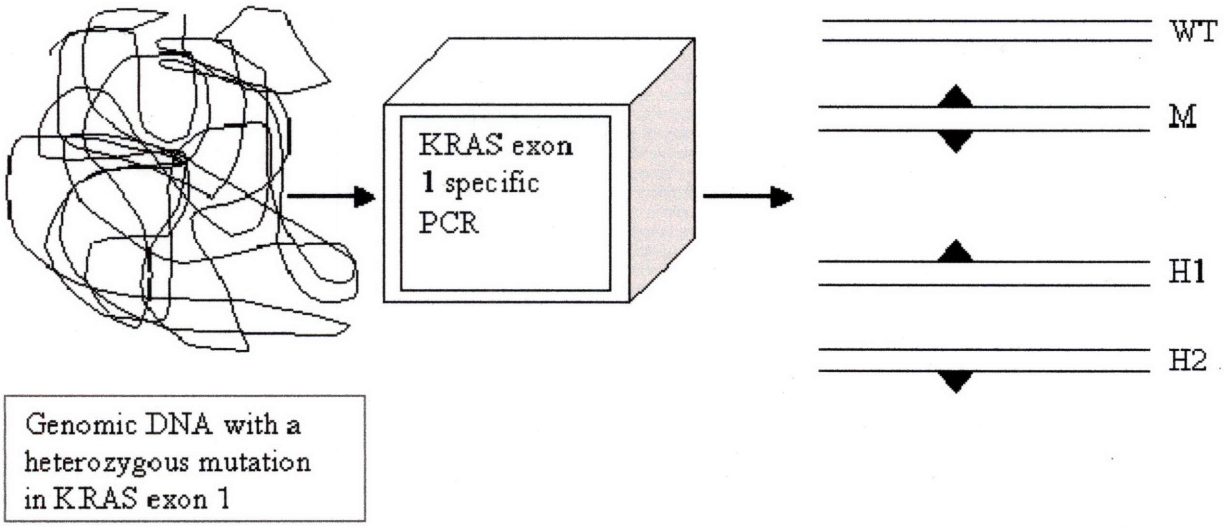
Figure 5: Screw Flexure to be used in this experimental apparatus.

2.2 Constraints and Functional Requirements

The screw flexure was meant to be designed to have translational deflections on the order of about one inch and rotational deflections of about twenty degrees for approximately four pounds of applied force. The sensors and data collection apparati must have resolutions on an order of at least 10 times more sensitive than the limits listed above in order to be able to detect meaningful trends.

upon the observation that double-stranded DNA fragments undergo melting into single-stranded DNA when exposed to denaturing agents, such as high temperature or a chemical denaturant (e.g., urea or formamide), as introduced by Fischer and Lerman [16-17]. Since the melting of a DNA fragment is dependent on both nucleotide sequence and the length of the fragment, two fragments, only differing by a nucleotide substitution, will reveal different melting profiles [16-17]. In denaturation followed by reannealing, four different species will form: one double-stranded wild-type homoduplex, one double-stranded mutant homoduplex, and two heteroduplexes (**Figure 1)** [26, http://rodium.no/srg]:

Figure 1:



Schematic illustration of the amplification of a heterozygous KRAS exon 1 samples. During PCR the continuous denaturation and re-annealing of single strand molecules allows the formation of four different species, one homozygous wild type (WT), one homozygous mutant (M), and two heterozygous (H1, H2) variants.

Under properly selected partially-denatured conditions, different forms of wild-type and mutant fragments and heteroduplexes will exhibit alternative secondary structures that will

allow separation in a sieving matrix during electrophoresis. However, if the two strands of the DNA fragment are forced to stay in proximity to one another during the partial-denaturing process, the separation performance can be enhanced further. This process can be achieved by extending the fragment at one end with a high-melting domain, such as an artificial GC-rich sequence (GC clamp) as described by Myers *et al.* [52-53].

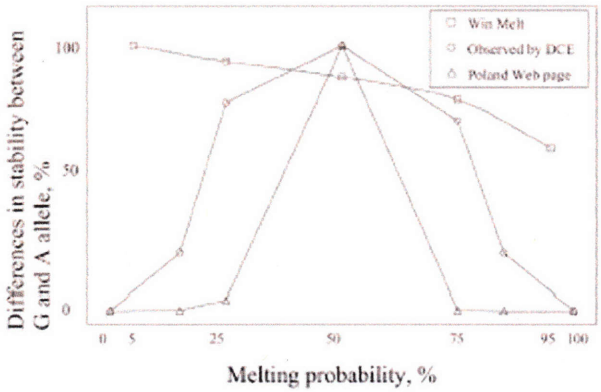
2.1.2. Poland's Algorithms, DNA Melting Profiles and Computer Programs

Poland's calculation of DNA melting profile algorithm [45] with Fixman-Freire approximation [46] is utilized as a theoretical method to calculate the thermal stability of a double-stranded nucleic acid (dsDNA) [47]. Based on algorithms, computer software programs like SQHTX, WinMelt, MacMelt, and the Internet web site Poland Algorithm (http://www/biophs.uni-duesseldorf.de/local/POLAND/poland.html), the melting profiles of defined double-stranded nucleic acid can be calculated.

The change in the structure of DNA from an orderly helix to a disordered structure without base paring is termed "melting." The computer programs calculate the midpoint temperature when each base pair is at a 50/50 equilibrium between the helical and melted states. The data are subsequently plotted as midpoint temperature versus the base sequence, creating a melting profile for the fragment [39]. Prior to denaturant capillary electrophoresis, the main purpose of creating melting profile analyses of DNA fragments using computer programs is to select and manipulate target sequences so that the region of interest can be in the low-melting domain.

Sample **Figure 2** below displays an illustration of thermodynamic differences obtained with both the Poland algorithm homepage and WinMelt calculation [39]:

Figure 2:



Relative differences (y-axis) in stability between G and A alleles in lymphotoxin alpha at different melting probabilities (*x*-axis) are shown. The thermodynamics calculated by the use of the Poland web page (△) predicted the observed stability difference between the alleles (○) at 50% melting probability. WinMelt (□) predicted difference in separation at all probability levels. At 50% melting probability, a good theoretical prediction of observed thermodynamic differences was obtained with both the Poland home page and WinMelt. This level of probability was used for further analyses.

(with permission)

2.2. Slab Gel Techniques

There are several different melting gel techniques. Each one is named according to the combination of denaturants in the gel (slab gels) or matrix (capillary platform), and the platform on which the analysis is performed. In slab gel techniques, the most noted ones are denaturant gradient gel electrophoresis (DGGE) [16-17], constant denaturant gel

measured. The second dial indicator measured the vertical translation due to the rotation of the upper stage.

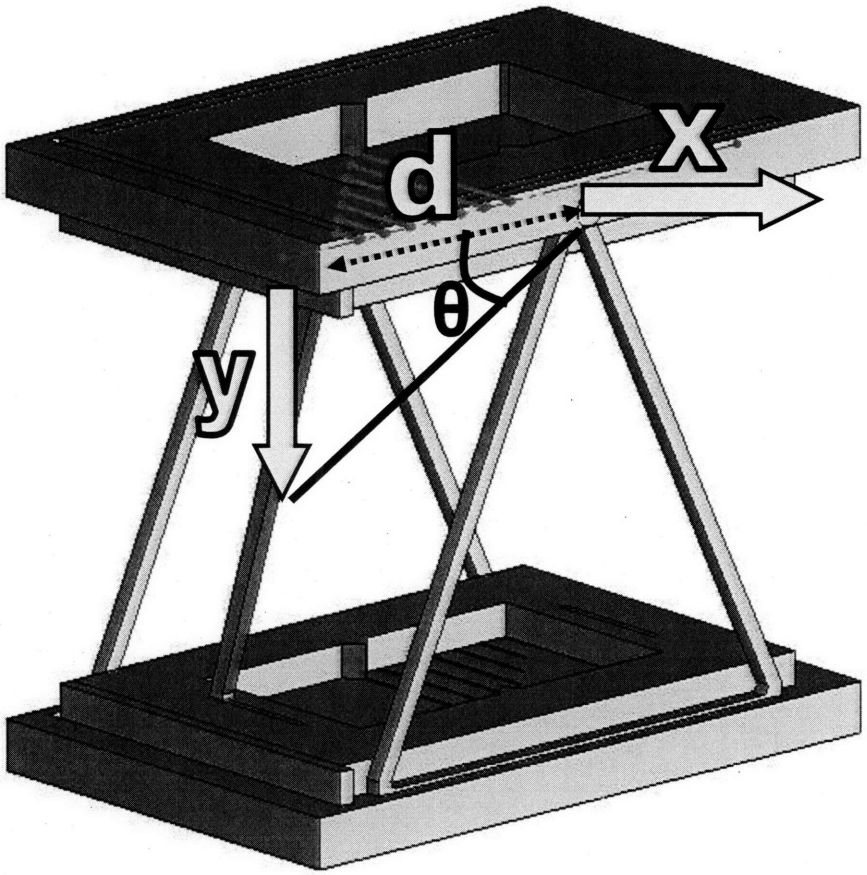


Figure 9: Diagram of geometric measurement techniques for Dial Indicators

Where d is the variable distance between the axis of rotation and the location of the side Z constraint piece, y is the vertical distance the edge of the stage moved, θ is the angle about which it rotated, and x is the horizontal distance the stage moved forward.

The angle of rotational displacement θ, was found by taking the inverse sine of the quotient of the vertical displacement over the arm length between the axis of rotation and the edge piece of the flexure as in Equation 2.

$$\theta = \sin^{-1}\left(\frac{y}{d}\right) \tag{2}$$

The distance d, illustrated in Figure 9, was variable depending on where the sliding side constraint was positioned.

3 Experimental Setup

3.1 Description of Experimental Apparatus and Procedure

The experimental apparatus consisted of the screw flexure and the two dial indicators fastened to a baseboard with the necessary support structures. To apply force to the screw flexure, lead weights of sizes ¼ pound to 2 pounds were hung in various combinations from a string that was tied to a hook connecting to the screw flexure. The entire system is shown in Figure 10.

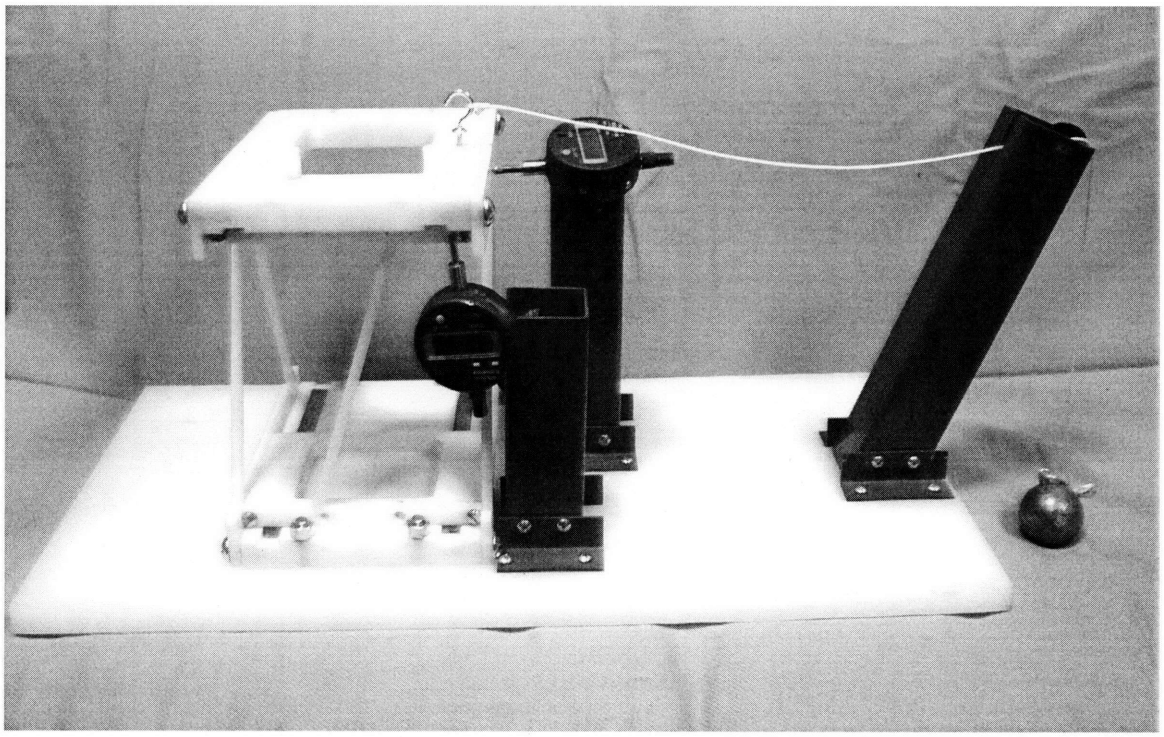
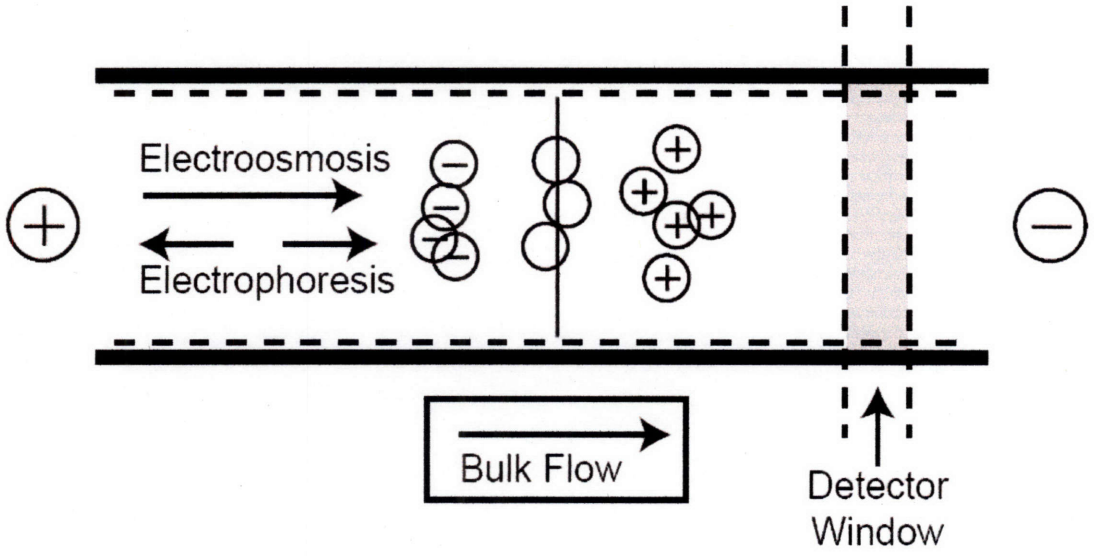


Figure 10: Photo of full experimental setup

DNA molecules in the solution are injected into the capillary by electro-kinetic or hydrodynamic injection. Separation inside the capillary takes place according to size under high-voltage conditions (5-30 kV) [42]. **Figure 3** below is a schematic illustration of EOF flow resulting from a charged inner capillary wall during application of an electric field [41, www.beckman.com/literature/Bioresearch]:

Figure 3:



A schematic of electrophoresis and electro-osmosis in a separation of anionic, neutral, and cationic analytes.

In a typical schematic CE instrument setup, the principal components include a PC computer for overall control, a power supply, a capillary passing through the optical center of a detection system connected to a data acquisition system, a sample introduction system, and an autosampler [42].

CE has proven to be an efficient and cost-effective analytical technique that allows for the separation of molecules of different nature and sizes contained in a small sample with high resolution.

2.3.2. Constant Denaturant Capillary Electrophoresis (CDCE) and Automated Denaturing Capillary Electrophoresis (ACDCE)

As described above, constant denaturant capillary electrophoresis (CDCE) is based on the same separation principles that underlie both denaturant gradient gel electrophoresis (DGGE) [16-17] and constant denaturant gel electrophoresis (CDGE) [22] with capillary electrophoreses (CE) of DNA in a linear polyacrylamide matrix [23]. The CDCE separation principle and method was first developed on a laboratory assembled instrument by the MIT and Northeastern University group in 1994 [6], and later adapted to commercially available capillary DNA sequencing instruments.

Figure 4 shows a simple diagram of the CDCE apparatus, adapted from a CE instrument for separation of DNA sequencing products [11]. The components are: a computer, a power supply, laser beam, narrow band-filter, mirror, glass lens, a detection window of a horizontally positioned fused-silica capillary, microscope object, 2^{nd} filter, a photomultiplier, a preamplifier, a current preamplifier, a data acquisition system, two buffer reservoirs positioned at each of the two ends of the capillary, a water jacket surrounding the cathodic end of the capillary, and a constant temperature water circulator to control the temperature [11].

Figure 4:

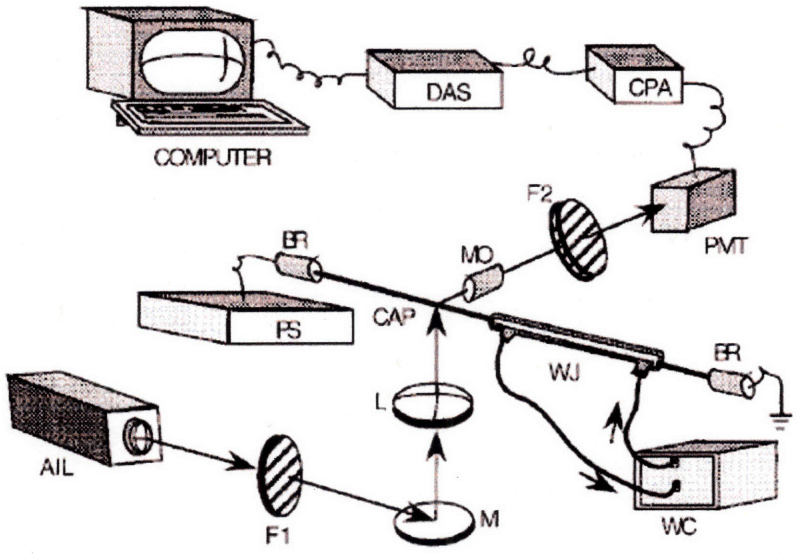


Diagram of the CDCE apparatus. The power supply supply (PS) is 30 kV dc (Model CZE 1000R-2032; Spellman, Hauppauge, NY). A laser beam from an argon ion laser (AIL) (Model 5425ASL; Ion Laser Technology, Salt Lake City, UT) is filtered through a 488 nm narrow bandpass filter (F1; 10 nm bandwidth; Corion, Franklin, MA), reflected by a 45° mirror (M; Newport, Irvine, CA), and focused by a plano-convex glass lens (L; Newport) onto a detection window of a horizontally positioned fused-silica capillary (CAP). Fluorescence emitted from the sample is collected by a 60 × microscope objective (MO; Newport) and passes through a 540 nm and a 530 nm filter (F2; Corion) into a photomultiplier (PMT; Oriel Instruments, Stratford, CT). The signal from the photomultiplier is amplified (10^7 or 10^8 V/A) by a current preamplifier (CPA; Oriel), recorded by a data acquisition system (DAS; ACM2-16-8A/T51B; Strawberry Tree Computers, Sunnyvale, CA), and transmitted to a Macintosh computer. The buffer reservoirs (BR) are positioned at the two ends of the capillary. At the cathodic end, the capillary is inserted into a water jacket (WJ), the temperature of which is controlled by a constant temperature water circulator (WC).

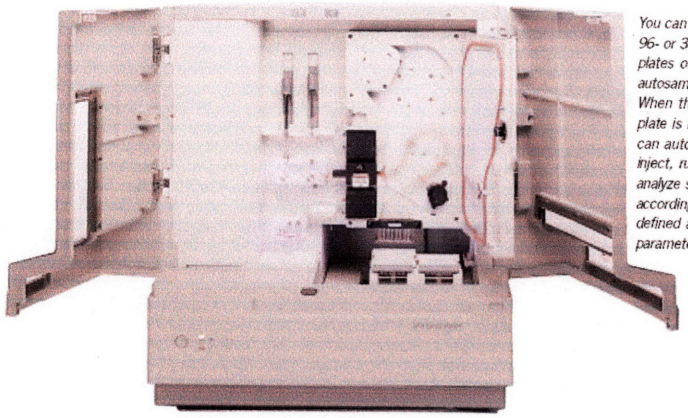
(with permission).

Automated denaturant capillary electrophoresis, or ACDCE, utilizes conventional commercially available capillary instruments as a tool to analyze mutations and SNPs by denaturant capillary electrophoresis [34]. It is based on the same separation principal as in DGGE described by Fischer and Lerman, CDGE, and TTGE. By the advantage of its automation process, ACDCE has achieved rapid analysis of a large number of samples over a short period of time. It has been used in the detection of mutations in *KRAS* Exon 1 [34] or *TP* 53 Exon 5-8 [43], as previously described.

Several capillary electrophoresis sequencing instruments are available from different manufacturers and have been utilized for this method: ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, USA), MegaBACE™ DNA Analysis System (Amersham Bioscience, USA), SEC9610 (Spectrumedix, USA), CEQTM 8000 Genetic Analytic System (Beckman Coulter, Fullerton, CA), and Agilent 2100 Bioanalyzer (Caliper Technologies, Mountain View, CA). Below, three of these instruments are illustrated in **Figure 5** [54]:

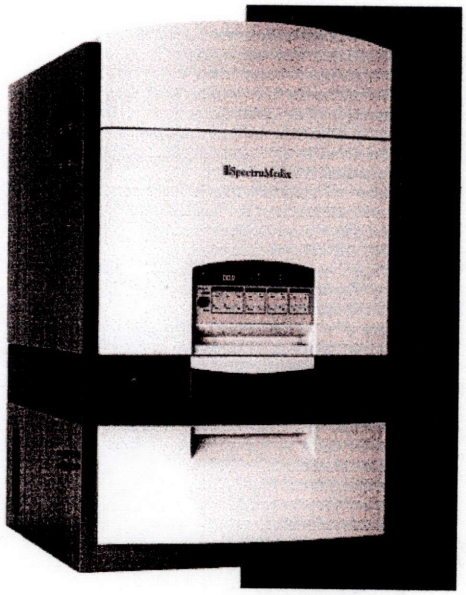
Figure 5:

ABI PRISM 3100 Genetic Analyzer:



You can place two 96- or 384-well plates on the autosampler at once. When the sample plate is in place, you can automatically inject, run, and analyze samples according to user-defined analysis parameters.

SCE9610:



MegaBACE System and Components:

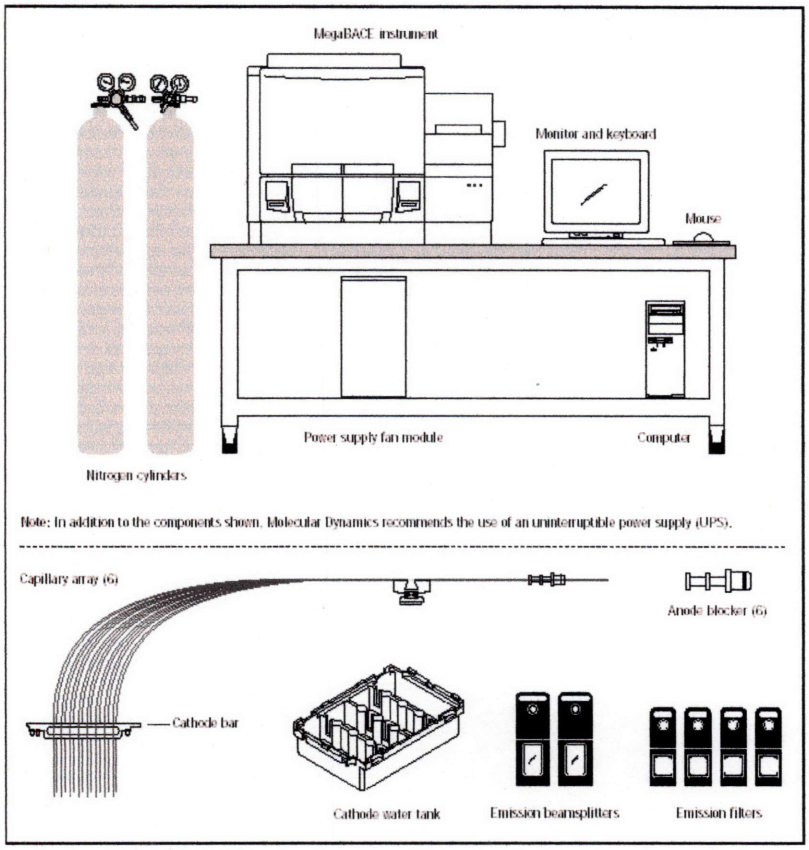


Figure 1-1. The MegaBACE system and components.

Chapter 3

CDCE Separation Theory and Protocol

The theory of point mutation separation using CDCE is based on differences in the melting temperatures [8-9]. The following flow diagram indicates the sample handling steps necessary to detect point mutations at different fraction levels [8].

3.1. Diagram for CDCE Separation Theory and Specific Protocol

In principle, these nine steps represent an outline for a procedure that is applicable to all DNA sequences, including those without a neighboring natural clamp. However, in the sequence without a neighboring natural clamp, an artificial clamp attachment is added in Step 4. For mutation detection at fractions as low as 10^{-6}, include Steps 1-3 and 5 prior to the performance of Steps 6-9. For mutation detection at fractions as low as 5×10^{-4}, include Steps 1 and 6-9. The steps are shown below in **Figure 6** [8].

Figure 6

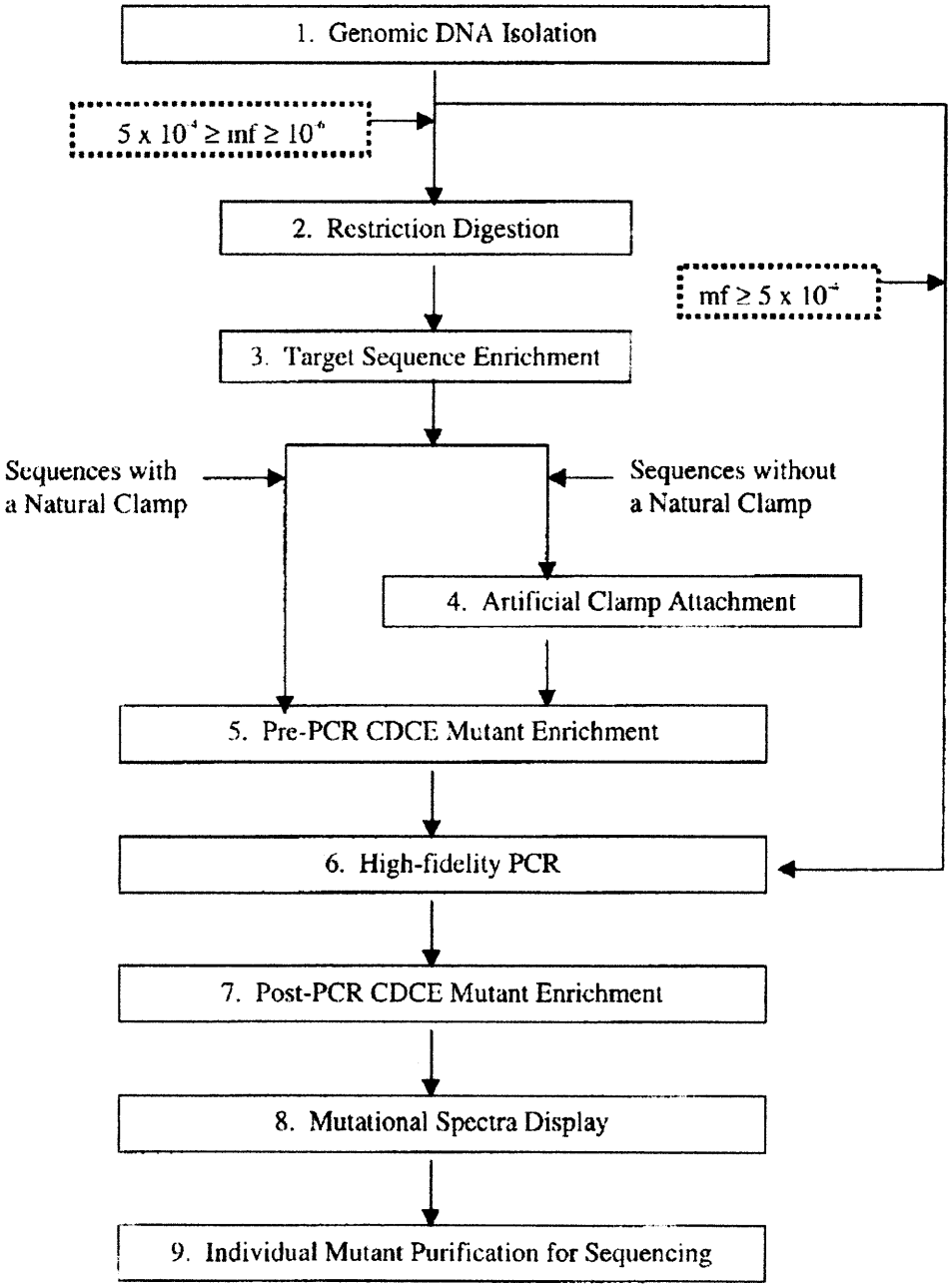


Figure 6: Flow diagram of the sample handling steps necessary to detect point mutations at different fraction levels. This procedure can be used for all DNA sequences, including those without a neighboring natural clamp. An additional procedure, Step 4, is introduced for DNA sequences without a natural clamp. For mutation detection at fractions down to 5 x 10^{-4}, include Steps 1 and 6-9. For mutation detection at fractions as low as 10^{-6}, include Steps 1-3 and 5 prior to performance of the steps necessary for mutation detection at fractions at or above 5 x 10^{-4} *(with permission)*

3.1.1. Fraction Level Detection of Point Mutations

As indicated in the diagram above, the specific protocol for measuring point mutations depends on the sensitivity of fraction level detection. To analyze the point mutations of fractions as low as 5×10^{-4}, the specific protocol begins with the isolation of genomic DNA either from blood or from solid tissues (Step 1)—for tissue samples, steps 1-4 are needed, for blood samples, steps 5-6 are needed. It is followed by the amplification of the target sequence with a high-fidelity DNA polymerase (Step 6), and then the post-PCR CDCE enrichment of mutant sequences relative to the wild-type sequences (Step 7). The actual separation of mutants in the enriched mixture occurs in the portion of the capillary heated by the water jacket connected to a constant temperature circulator, and is measured by CDCE (Step 8). Finally, in the last step (Step 9) the individual mutants complete a purification and sequencing process for their eventual identification. Kim *et al.* have pointed out that this method has demonstrated sensitivity in the following three types of applications: (1) the detection of point mutations occur in phenoltypically changed human cells or organisms after chemical treatment [8], (2) the detection of cancer cells in normal human tissues [29, 8], and (3) the identification of SNPs in pooled human blood samples [3, 8].

On the other hand, the protocol for the analysis of point mutations at fractions down to 10^{-6} needs three additional steps prior to Step 6's high-fidelity PCR. These three steps are: restriction digestion (Step 2), enrichment of target sequence from restriction-digested DNA (Step 3), and pre-PCR mutant enrichment by CDCE [8]. The sensitivity of this method has been used in applications with chemically treated human cells without

reference to phenotypic selection [7, 10]. For instance, Khrapko *et al.* reported in 1997 that they developed a method that allowed the observation of point mutations at fractions at or above 10^{-6}, which resulted in observing the hotspot point mutations that lie in 100 base pairs in human mitochondrial DNA [7].

3.1.2. Natural Clamp and Artificial Clamp

Step 4 of the flow diagram shown above is an additional procedure that is introduced for DNA "sequences *with* a natural clamp" or "sequences *without* a natural clamp" options [8].

Kim *et al.* state that the use of partial denaturation for DNA separation requires that a target be a DNA sequence domain (≈ 100 bp) juxtaposed with a domain of a higher melting temperature called a "clamp." If a certain 100-bp sequence is juxtaposed with an internally occurring clamp (a natural clamp) in genomic DNA, it can be analyzed directly using CDCE; however, when analyzing sequences without a natural clamp, an externally attached clamp by PCR is required [53] to detect point mutations at fractions down to 5 x 10^{-4}. Unfortunately, the attachment of the clamp to PCR produces polymerase-created mutations that can prevent mutation detection at fractions below 5 x 10^{-4} [8]. To remedy this problem, Kim *et al.* developed a ligation-based artificial clamp attachment technique in Step 4 to extend the detection capacity to measure point mutations at fractions as low as 10^{-6} [8]. Furthermore, in their 2003 published study, Kim and Thilly's clamp ligation-added method reported can increase the scanning range to an additional 89% of the human genome (in addition to CDCE/hifiPCR's applicability to only 9% of the human genome), and can open up more than 90% of the exons in known human genes

(http://pubgeneserver.uio.no/PubGene/tools/MeltMap/Index.cgi) to rare mutational analysis and pool DNA samples [59].

3.2. Remarks on CDCE Method Validation

The simple nine-step protocol discussed above has demonstrated CDCE is an ideal separation tool for the analysis of DNA variations and mutation detection in large sample material, as well as for identifying and isolating point mutations. Its validation lies in the capacity to exhibit the strength of the CDCE method, such as: its sensitivity for detection (including both qualitative and quantitative parameters), LOD capacity (lowest level detection limit), specificity (e.g., the ability to detecting a wild-type sample as a wild-type sample, not a mutant), repeatability (referring parallel and serial analysis of the same samples), and throughput (analyzing the number of samples in a defined time range) [5]. Scientists and CDCE researchers, including Bjorheim, Ekstrom, Karger, Khrapko, Li-Sucholeiki, Minarik, Thilly *et al.* have demonstrated repeatedly that the technological development of CDCE from single, custom-assembled instruments onto modified, commercial multicapillary instruments with an automated fraction collector has ushered CDCE to the front line for use in large-scale genomic research [5]. This chapter, along with the theoretical underpinnings and applications of this methodology, has paved the way for the research to be described in the following chapters on fraction collection using CDCE.

Chapter 4

Fraction Collection: Concept and Earlier Designs

4.1 Fraction Collection: Concept

In principle, after applying DNA separation techniques, further analysis of the separated components of a sample is necessary in order to obtain complete, detailed information on the fragment components. The fraction collection system has the capability to further analyze these separated fragment components using specific secondary reactions for identification and purification of complex mixtures. Khrapko *et al.* discussed the advantages of using this method for further determination of specific mutations in their report [6]. To have the capability to perform multi-dimensional separation makes a fraction collection system a high-throughput preparative separation system, which has the advantage of allowing for the collection of every component of a given sample mixture for further fragment purification and quality identification.

There are various micropreparative techniques for fragment purification and fraction collection, such as electrophoresis and chromatography. Based on the CE principle, the main components for a fraction collection system are: a sample section, a separation section, a detection section, and a fraction collection section.

In the past, several CE fraction collection designs were introduced and used in either a single-capillary or multicapillary format. In general, commercial multicapillary DNA analyzers are not equipped with automated fraction collection capabilities [4]. Fully

automated single-column systems are only available for fractionation and collection of specific sample components for a given run [4].

4.2 Fraction Collection: Early Designs

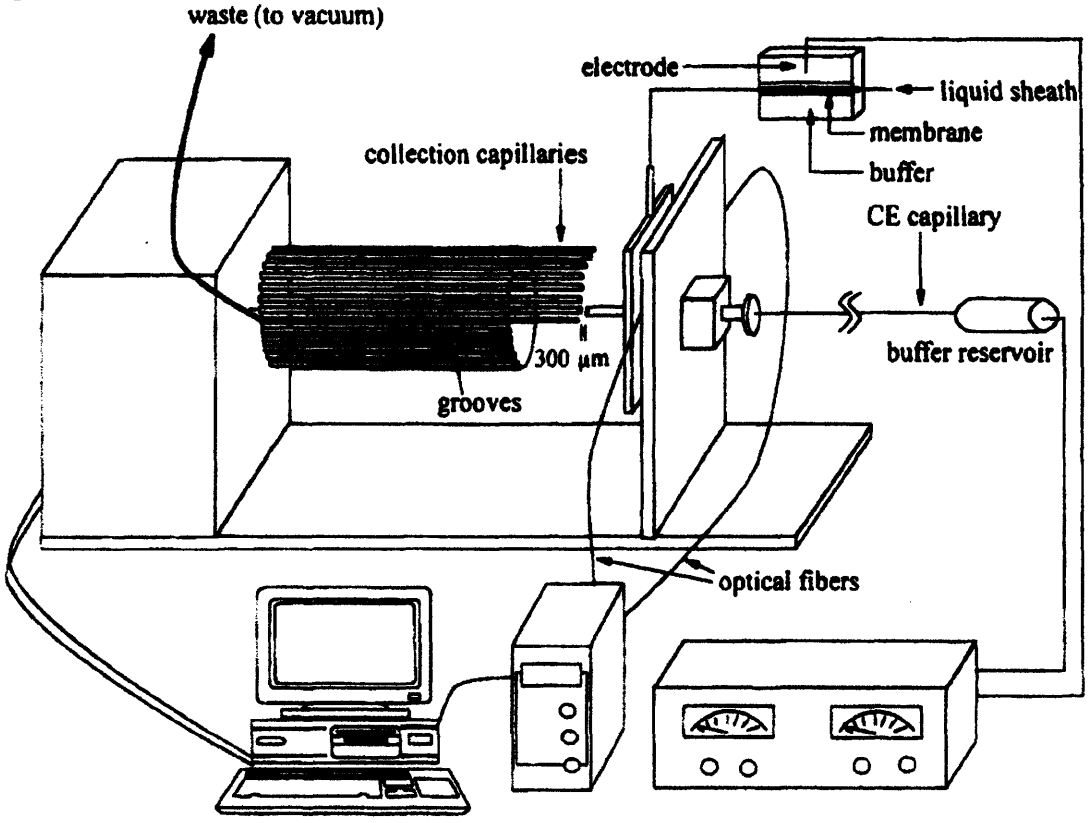
Several early CE fraction collection designs have been used in the past. This study will briefly review a few representative devices and their innovations. The review will be divided into two parts: fraction collection in single-capillary electrophoresis systems and fraction collection in multicapillary electrophoresis systems.

4.2.1. Single Capillary Electrophoresis Systems

4.2.1.1. Fraction Collector by Muller *et al.*, 1995 [63]:

O. Muller *et al.* stated in their research report: this fraction collector was an automated, high-precision device suitable for any mode of CE. This device was the first to utilize detection close to the end of the capillary and a sheath liquid at the exit of the capillary to allow for the continuous collection of multiple fractions without interruption by the applied electrical field. **Figure 7** below presents a schematic view of the sheath flow collection device [63]:

Figure 7:



Schematic view of the sheath flow collection device [62] *(with permission)*

This fraction collection device consisted of two portions: (1) the detection interface unit (including a fiber-optic detection cell and sheath flow unit), and (2) the fraction collector (including a holder of collection capillaries). The collection reproducibility was up to 60 fractions of a microliter, which could be automatically collected into capillaries used as collection vials during a single CE run without interruption from the electric field. Continuous operation and high collection precision were achieved by detecting the migrating zones close to the end of the column using an optical fiber-based UV detector [63].

4.2.1.2. Fraction Collection in CZE and CIEF by Minarik *et al.*, 2000 [65]:

This high-resolution capillary fraction collection system, designed by Minarik *et al.*, could be applied to both capillary zone electrophoresis (CZE) and capillary isoelectric focusing (CIEF). Two-point detection was the design feature on this device used to determine zone velocity close to the column end for accurate estimation of zone exit time. To increase the amount of the injected sample, they used 200 μm ID capillaries for CZE and 100 μm ID capillaries for CIEF. In order to prevent undesired liquid flow, they closed the separation system at the inlet with a semipermeable membrane. Finally, low conductivity buffers were employed in CZE and a slowly increasing, focusing voltage in CIEF reduced Joule heating. **Figure 8** below presents the principle parts of this micropreparative instrument [65]:

Figure 8:

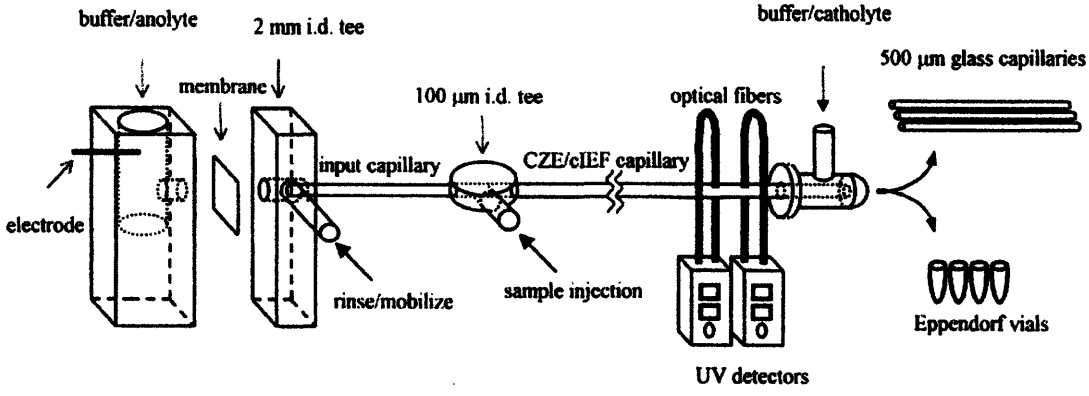


Diagram of a fraction collector for capillary array electrophoresis [65] *(with permission)*

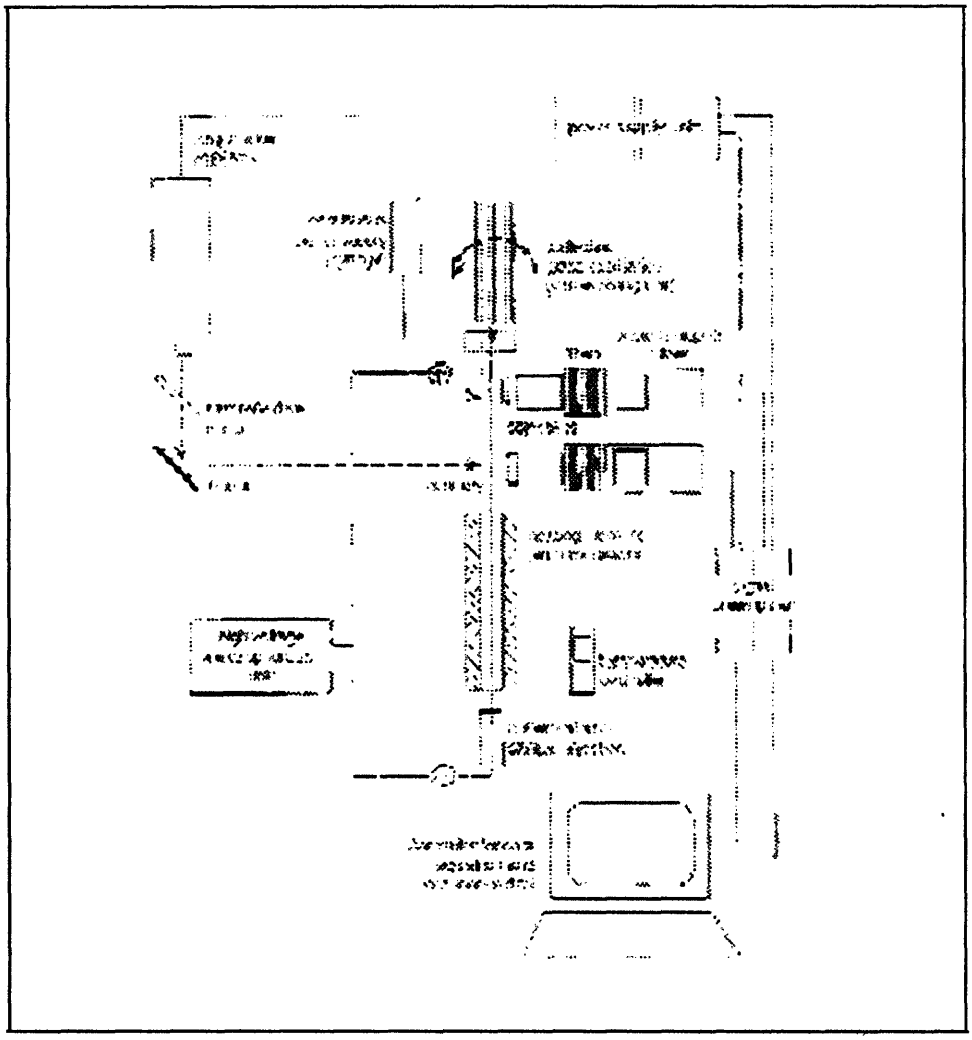
4.2.1.3. Two-point fluorescence detection and automated fraction collection applied to constant denaturant capillary electrophoresis by Ekstrom *et al.*, 2000 [28]

Ekstrom *et al.* upgraded and modified a commercially available, single-capillary CDCE instrument using two-point fluorescence detection with automated fraction collection to detect point mutations in DNA sequences of 100 bp lengths with frequencies as low as 10^{-4}. Fraction collection was integrated into the CDCE instrument. Similar to Muller's device, sheath liquid flow at the exit of the capillary allowed continuous collection of multiple fractions with an uninterrupted applied electrical field. Up to 60 fractions of small volumes could be automatically collected like Muller's [63].

Ekstrom *et al.* stated that the two-point detection was achieved by using a beam splitter to divide the filtered laser beam from the low-power argon laser into two beams. The significant feature of the two-point detection was that it could predict the elution time of peaks and then calculated the elution time needed to collect fractions accordingly [28].

In order to identify an unknown mutant, a second collection was needed, followed by PCR and sequencing of the PCR product. The second approach involved verifying the mutations by hybridizing the sample with standard mutants having similar peak patterns in the electropherogram, followed by reanalysis using CDCE. Since the mutant fraction of the sample used was 10^{-4}, detected mutations could not easily be distinguished from errors if the analyzed mutant fraction was below 10^{-4} [28]. **Figure 9** below shows the instrumental setup for the two-point detection CDCE [28].

Figure 9:



Schematic drawing of the instrument setup for the two-point detection CDCE *(with permission)*

4.2.2. Multicapillary Electrophoresis Systems

In the following sections, three fraction-collection designs by Irie (2000), Minarik (2002), and Berka (2003) are discussed.

4.2.2.1 Automated DNA Fragment Collection by CAGE by Irie *et al.*, 2000 [67]

Figure 10:

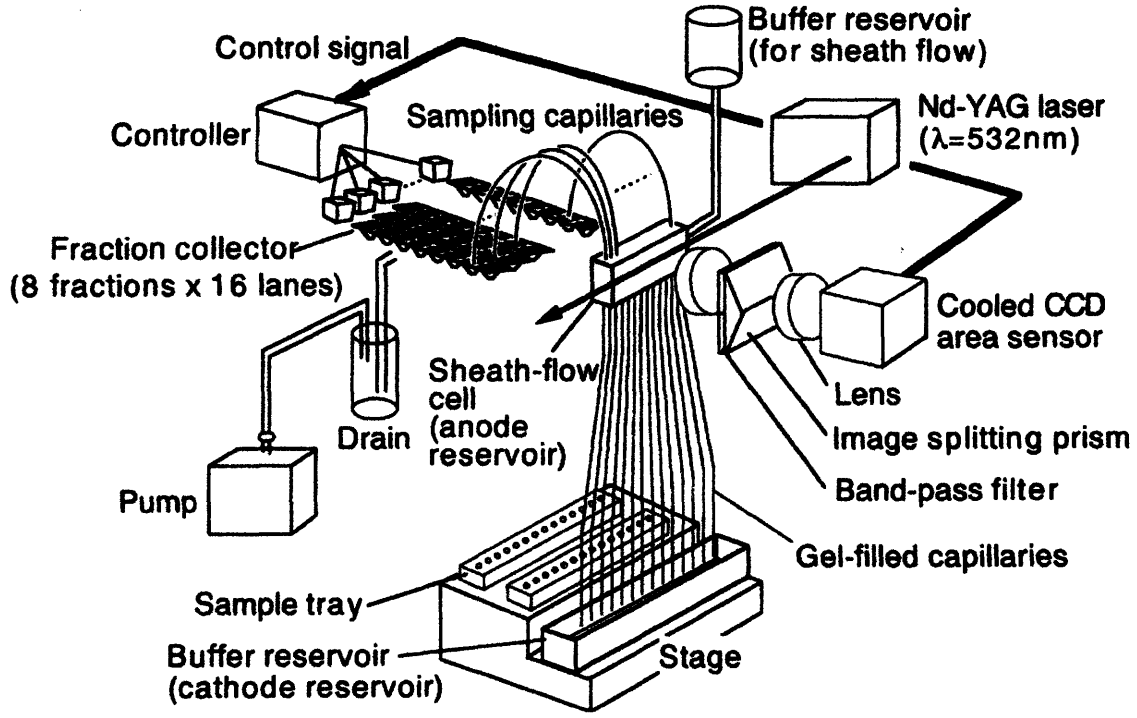


Figure 10: Schematic drawing of the automatic DNA fragment collector *(with permission)*

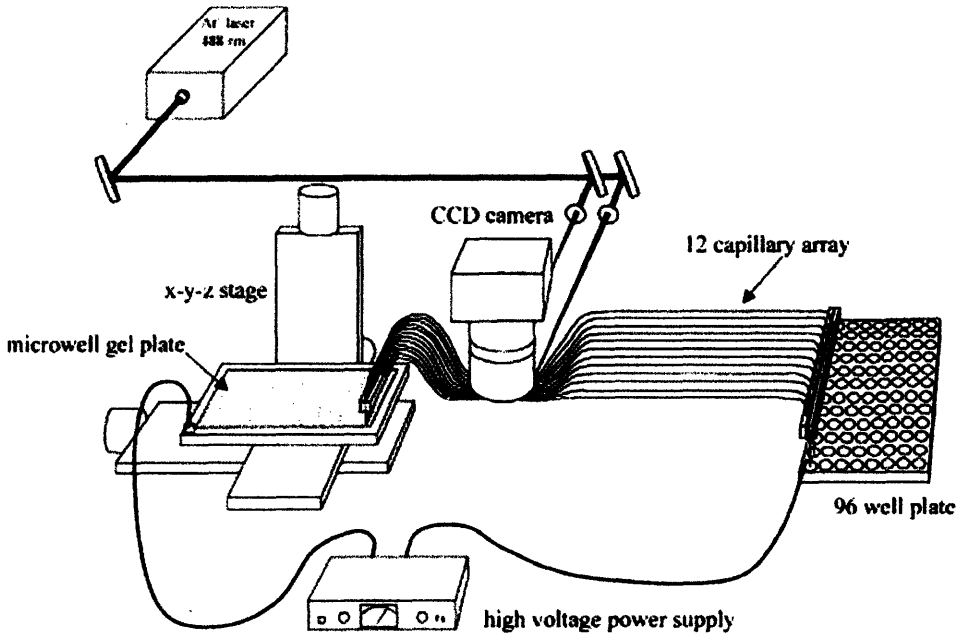
Irie's automated DNA fragment collection shown in **Figure 10** was based on capillary array gel electrophoresis (CAEG). A sheath flow technique was used for both mutation detection and DNA fragment collections. Sixteen gel-filled capillaries transported the DNA fragments to a fraction collection consisting of 16 identical lanes. By applying negative high voltage, DNA fragments migrated in the gel-filled capillaries from the buffer reservoir (cathode reservoir) to the sheath flow cell (anode reservoir). They were then transported from the sheath flow cell to the vials set on the fraction collector through the sampling capillary. Multiple fluorophores attached to different DNA fragment

groups were detected by using an image-splitting prism combined with band-pass filters and a cooled CCD area sensor [67].

4.2.2.2. Design of a Fraction Collector for CAE by Minarik *et al.*, 2002 [68]

Minarik *et al.* designed a prototype instrument in 2002 for high-throughput fraction collection with capillary array electrophoresis (CAE) with two-point detection and side-entry illumination [68]. As described in the report, the design of the system was based on a comprehensive collection approach: (1) fractions from all capillaries were simultaneously collected in an individual collection microwell, which eliminated the need for multiple controls of individual microwell collectors in predefined time intervals; (2) the collection time intervals were based on the estimated widths of the separated peaks; (3) the location of the fraction in the microwells on the collection plate was determined based on zone velocities measured between two detection points; (4) different from Muller's sheath liquid, the collection microwell plate was fabricated from buffer-saturated agarose gel, which resulted in the maintenance of uninterrupted electrical contact with the separation capillaries during the collection process; (5) liquid evaporation from the collection well was kept to a minimum due to the fact that the collection gel plate was made of over 90% water; (6) collection performance was demonstrated by reinjection of selected fractions of a double-stranded DNA separation; and (7) the identity of these DNA fragments was confirmed by PCR and sequencing for further analysis [68]. See **Figure 11** below:

Figure 11:



Design of a capillary array instrument with two-point detection and side-entry illumination for high sensitivity and high-resolution multicapillary fraction collection applications [68] *(with permission)*

4.2.2.3. Design of an Automated Fraction Collection for CAE by Berka *et al.*, 2003 [69]

Berka *et al.* designed this high-resolution, capillary array electrophoresis with automated fraction collection for the GeneCalling analysis of yeast genomic DNA in 2003 [69]. This instrument integrated a 12-capillary array for DNA separation with a replaceable sieving matrix and agarose-based collection plate, laser-induced fluorescence detection, and an automated microfraction collector. Berka *et al.* reported that: (1) the DNA fractions exiting the separation capillaries were continuously deposited in a 1536-well collection plate made of agarose gel; (2) DNA fragments recovered from selected fractions were cloned and then sequenced; (3) over 80% of theoretically predicted fragments could be recovered in the collected fractions, cloned and sequenced with an

average three-fold redundancy; and (4) the suitability of capillary array electrophoresis for DNA fragment recoveries was proved by the correlation of experimentally obtained sequences with theoretically predicted gene fragments.

In sum, since the fraction collection method is an important element for fragment purification and quality identification, these early designs in single- or multicapillary systems collectively have led to the development of the next design phase, created to meet the high standard of CDCE for sensitivity, robustness, and cost-effectiveness, as exemplified by the HTMS Model, which will be described in detail in the following chapters.

Chapter 5

The Instrument Design and Materials: HTMS Model

5.1 Instrument Design with Fraction Collection Module: HTMS Model

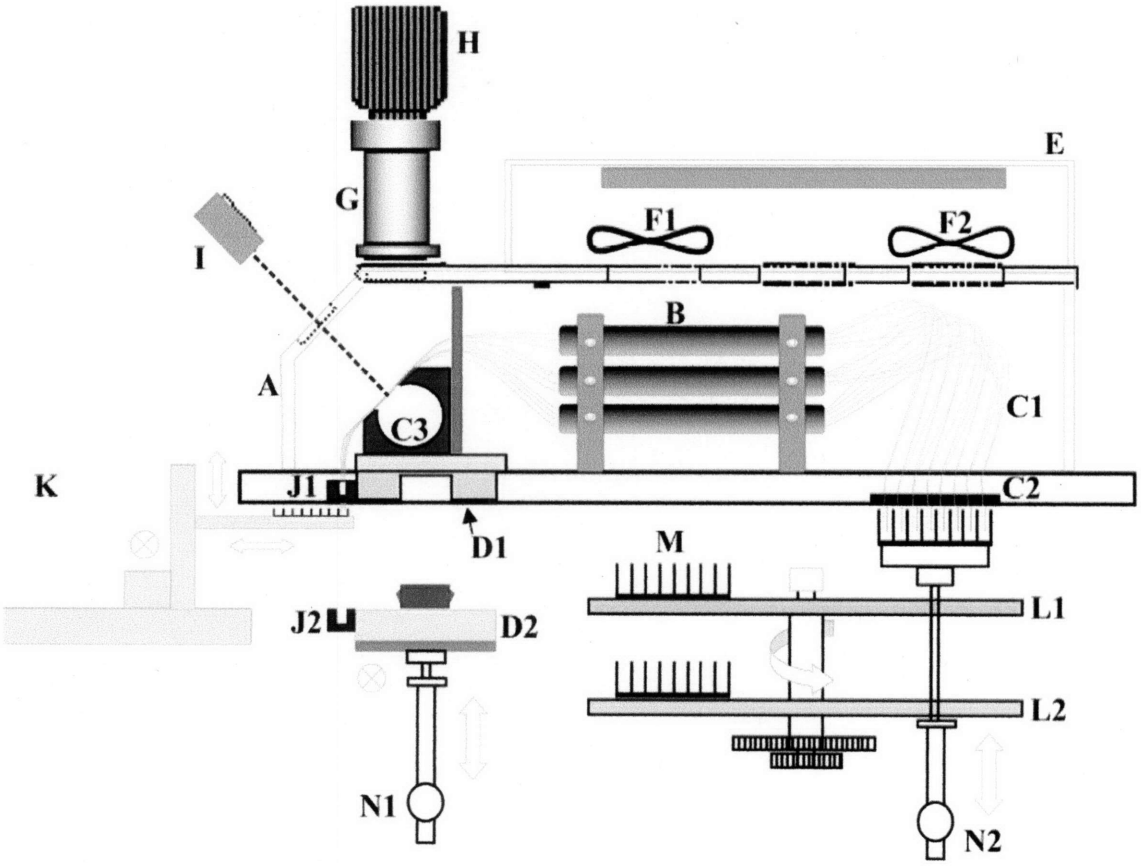
The High-Throughput Mutational Spectrometer (HTMS) instrument was constructed by modifying a SCE2410 24-capillary DNA sequencer (SpectruMedix, State College, PA) [61] into a fully-integrated, automated multicapillary CDCE instrument with fraction collection to detect low-frequency mutations in pool samples for large populations by Q. Li *et al.* [4]. In general, the SCE2410 shares the same basic layout and components as the SCE 96-capillary, 192-capillary, and 384-capillary instruments, since they all have the same instrument platform and similar capabilities (http://www.spectrumedix.com) [4, 61].

This HTMS represented an improvement over previous multicapillary instruments in the following three areas: 1) a substantial modification of the optical detection module in the SCE2410 24-capillary DNA sequencer, 2) addition of new modules for precise temperature control, automated matrix replacement and fraction collection; and 3) redesign of the multicapillary cartridge in order to accommodate these stated modifications [4].

The major components of the HTMS include a capillary cartridge containing an array of 24 capillaries (Polymicro Technologies, Phoenix, AZ) (A) weaving through a solid-state heater array, and ending in a 3 x 8 array for injection and a 1 x 24 array for

fraction collection. At the top, the cartridge is interfaced with an optical detection module (consisting of an argon laser, a scanner, a fluorescence collection lens and a CCD camera). At the bottom, the cartridge is interfaced with a matrix replacement module, a fraction collection module, and a sample delivery system. **Figure 12** is the schematic drawing of the HTMS instrument [4]:

Figure 12



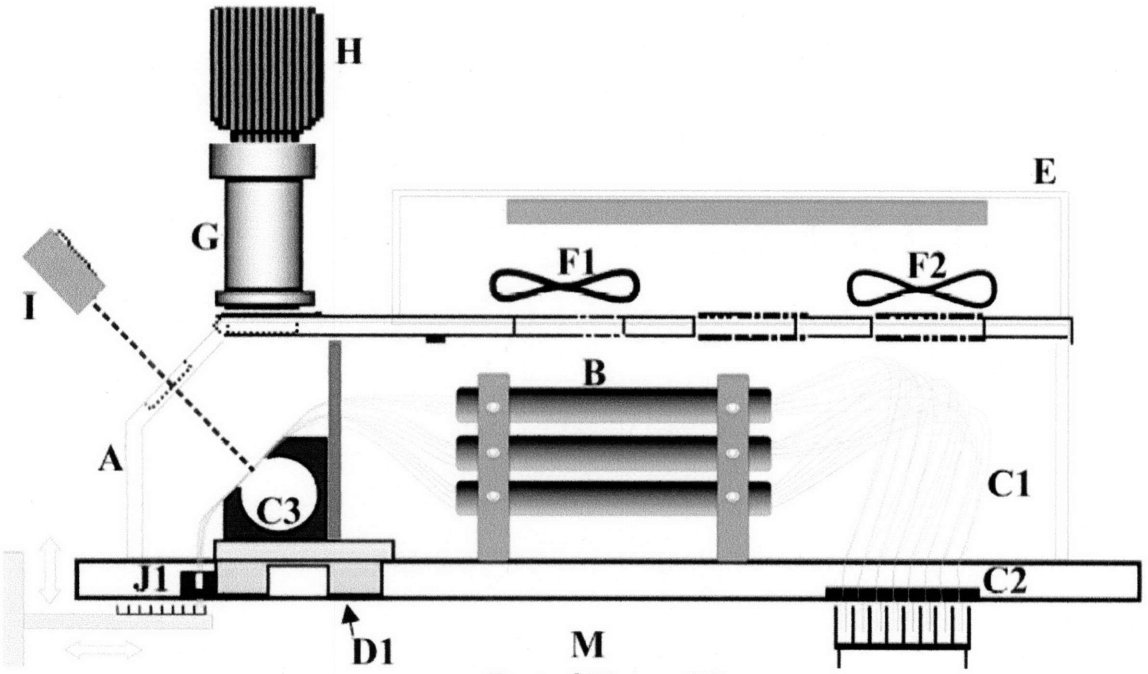
Schematic of the HTMS Instrument:
The instrument consists of a capillary cartridge (A), containing an array of 24 capillaries weaved through a solid-state heater array (B) and ending in a 3 x 8 array for injection and a 1 x 24 array for fraction collection. The cartridges interface at the top with an optical detection module consisting of an argon laser which beam is passed through a scanner (I), a fluorescence collection lens (G), and a CCD camera (H). The cartridge interfaces at the bottom with a matrix replacement module, a fraction collection module, and a sample delivery system (L1, L2, N2, M). The matrix replacement module comprises an automated docking system (D1, D2) to which is attached a high pressure chamber (J1, J2). The upper half of the docking system (D1) is fixed to the bases of the capillary cartridge and its lower half (D2) is mounted on a 2-axis pneumatic stage (N1). It provides both the in-out and up-down motion for D2. The horizontal in-out motion of the pneumatic

state is used to position and align the two halves of the docking system, and the vertical motion is used to dock the two parts together. The fraction collection module (K) comprises a 3-axis translation stage, which mounts an agarose tray. The sample delivery system consists of two programmable carousels (L1, L2) that carry up to seven 24-well sample trays. A pneumatic stage (N2) lifts sample and buffer trays of the carousels for injection and electrophoresis. The ambient temperature within the capillary cartridge is regulated by a heated air flow system (E). (*with permission*).

The following sections will address each of the instrument's major components, its set-up, and the functions of the capillary cartridge, optical detection module, matrix replacement module, and sample delivery system in conjunction with the fraction collection module [4].

5.1. 1. Capillary Cartridge

An array (C1) of 24 capillaries are enclosed in a removable cartridge (A) which includes an array of six independently-controlled, precision solid-state heaters (B), an integrated injection device with an array of 24 electrodes (C2), and a receiving socket for automated docking and undocking of a matrix replacement module (D1), as shown in the close-up from part of **Figure 12** below [4].



(Part of **Figure 16**)

The primary function of each of these six individual solid-state heaters is to regulate the temperature of a group of four capillaries weaving through four stainless steel tubes. Inside the cartridge, the ambient temperature is kept at 40.0 ± 0.3°C through regulated flow of heated air from a separate heater (E) positioned on top of the cartridge. Adjacent to the separate heater, two fans (F1 and F2) are installed to generate the air flow [4].

The injection ends of the 24 capillaries are laid out in a 3 x 8 array and the fraction collection ends are placed in a 1 x 24 array. The fused-silica capillaries, with an inner diameter of 75 μm and an outer diameter of 375 μm, are positioned 4.5 mm apart at the fraction collection end and 9 mm apart at the injection end. The total capillary length is 65 cm, with the detection window located at 50 cm from the sample injection end. To

perform the electrophoresis run, the required high voltage is applied via a 3 x 8 electrode array using gold-coated pins (Mill-Max, Oyster Bay, NY), which also allows real-time monitoring of the current going through each capillary [4].

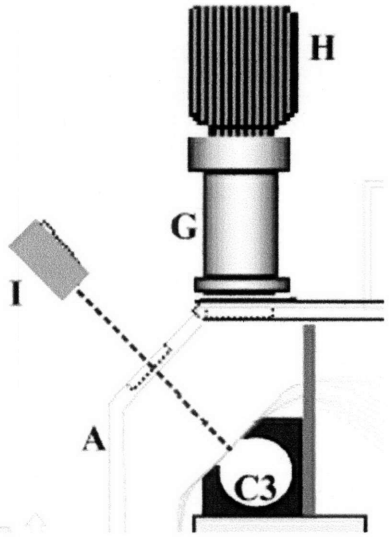
Inside the cartridge, a small section of about 4 cm of the capillaries arranged as a parallel array connected with an aluminum mounting piece (C3) to supply the optical window with illumination and detection capacity. A 1.5 cm section of polyimide coating is stripped off in order to expose clear capillary tubes at the optical window [4].

5.1.2. Optical System

The primary purpose for making such substantial modifications to the SCE2410 optical system in the HTMS is to improve sensitivity and dynamic range, and to reduce crosstalk. Accordingly, these modifications are incorporated into the instrument's array layout, imaging lens design, and wavelength selection [4]. This HTMS optical system includes a single-color measurement of fluorescence, the imaging lens, CCD camera system, optical filter assembly, and the power supply for the camera and laser as detailed in below.

In fact, the first modification is the reduction of the measurement of fluorescence to a single color, instead of ten-color (wavelength) units for four-dye chemistry in the SEC2410 DNA sequencer [61]. To enhance illumination, a galvanometric scanner (I) (Model G124, GSI Lumonics, Wilmington, MA) is used in the HTMS, which scans across the entire array with a 488-nm laser beam. A custom designed multi-element imaging lens (G) with a numerical aperture of 0.45 (Optics1, Manchester, NH) collect the laser-induced

fluorescence, which is then detected by a back-illuminated CCD camera (H) (Spec-10, Roper Scientific, Trenton, NJ) [4], as shown in the close-up from part of **Figure 12** below [4].



(Part of **Figure 16**)

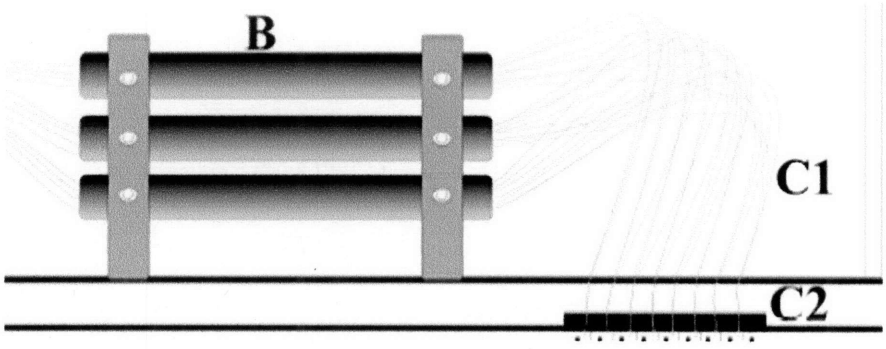
5.1.3. CDCE Temperature Control System ″

Six solid-state thermal heaters (B) are contained in the precision thermostat array for the CDCE temperature control system. Each solid-state heater is individually controlled to maintain a temperature accuracy of ±0.013°C [4].

Each heater contained a cylindrical block of copper, 6″ in length and 1″ in diameter, with a flexible heating element (Model HK5424, Minco, Minneapolis, MN) wrapped around it and a pair of thermistors (Model 46008, YSI Temperature, Springfield, NJ) permanently embedded in it. There are four through-holes parallel to its cylindrical axis,

through which four stainless steel tubes of 400 μm inner diameter are embedded. The thermistors are responsible for temperature sensing and feedback control. The removable precision thermistor (Model 5611, Hart Scientific, American Fork, UT) is responsible for monitoring the core temperature of the heater block [4].

Connected directly to the YSI thermistors and heating element, an analog temperature controller (Model TC7100, Vere Electronics, New Kensington, PA) helps achieve temperature control by monitoring and adjusting the voltage from the YSI thermistors to match a reference voltage sent to the computer. A software module written in LabVIEW (National Instruments, Austin, TX) monitors the absolute heater temperature [4].

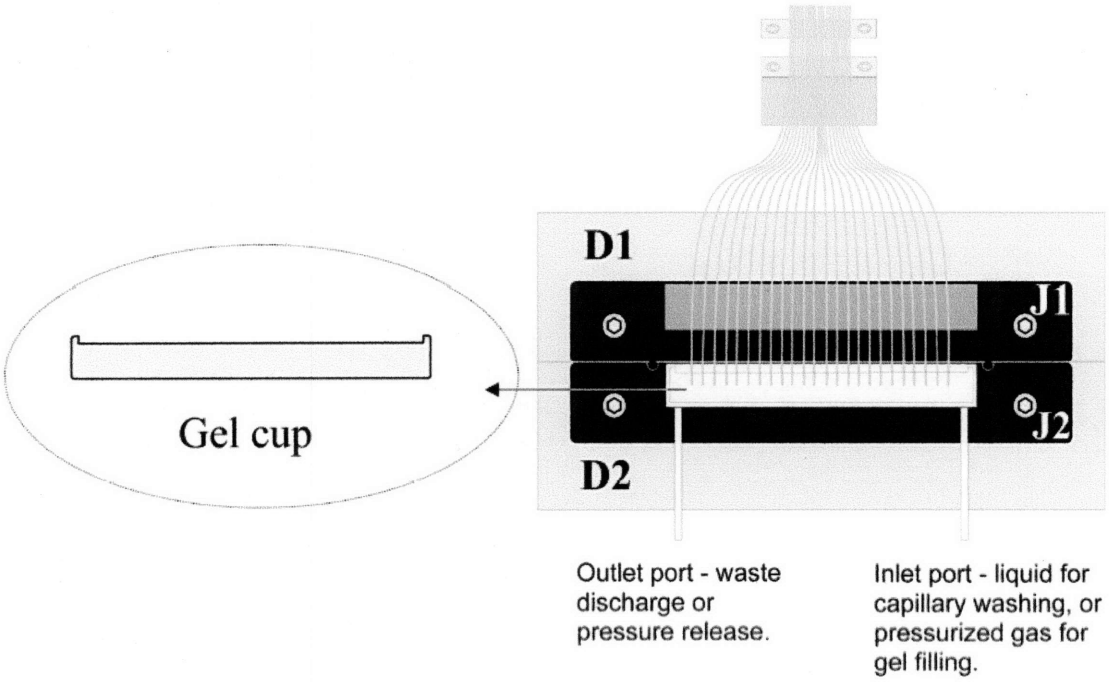


(Part of **Figure 12**)

5.1.4. Matrix Replacement

Figure 13 below is a schematic of the HTMS automated matrix replacement module [4].

Figure 13:



Matrix replacement module: The matrix replacement module consists of a high-pressure chamber (HPC) mounted on an automated docking system and connected to a high pressure helium tank. The HPC consists of two separate halves (J1, J2), each of which is mounted on a pair of mated parts of the docking system. The bottom-half of the HPC (J2) has an elongated cross-sectional cavity. A gel cup containing the matrix is placed in this cavity. *(with permission)*

This matrix replacement module consists of a high-pressure chamber (HPC), which contains two separate halves (J1 and J2). Each half is mounted on an automated docking system (D1, D2) (Model MXC160, Applied Robotics, Glenville, NY). A gel cup containing the matrix is placed inside the bottom-half of this high-pressure chamber, J2 [4].

In principle, matrix replacement occurs by enclosing the fraction collection end of the capillary array within a high-pressure chamber. Inside the chamber, both of these two

halves are enclosed in the same rectangular block of stainless steel with a slight difference: J1 with a flat-bottomed surface, and J2 with an elongated cavity with dimensions of 4.25″ (L) x 0.625″ (H) x 0.375″ (W). J2's function is to accommodate a gel cup during matrix filling or to serve as a reservoir for solution during capillary wash.

The gel cup with a matrix is placed in the elongated cavity to fill the capillaries with separation matrix (SpectruMedix, Catalog No. MSEQ-180-001). The two halves lock together and the chamber is pressurized to 1000 p.s.i. with compressed helium for 20 minutes, forcing the matrix through the capillary array. After electrophoresis and fraction collection, the two halves re-engage for capillary wash by alternating from the pressurized helium gas to a pressure-regulated HPLC pump that delivers a wash solution to the capillary array [4].

5.1.5. Fraction Collection

The design of this HTMS fraction collector module (K) contain a 1536-well (48 x 32) agarose tray that mounted on a XYZ translation stage (Custom ICS 3-axis system, IAI America, Inc., Torrance, CA). This agarose tray is made from a solution of 50% Metaphor and 50% Seakern Gold agarose in 1 x TBE buffer. The agarose tray is put in a Delrin tray, connect to the horizontal arm of an L-bracket mounted on the Z-stage. Placed between the Delrin tray and the L-bracket mounting base is a rotation stage. This rotating stage is designed to provide proper adjustment of rotational alignment called for between the rows of wells in the agarose tray and the 1 x 24 linear array of the fraction collection ends of the

capillaries. Two metallic pins are pierced into the agarose tray to provide grounding for the high-voltage electrophoretic circuitry [4].

(Part of **Figure 12**)

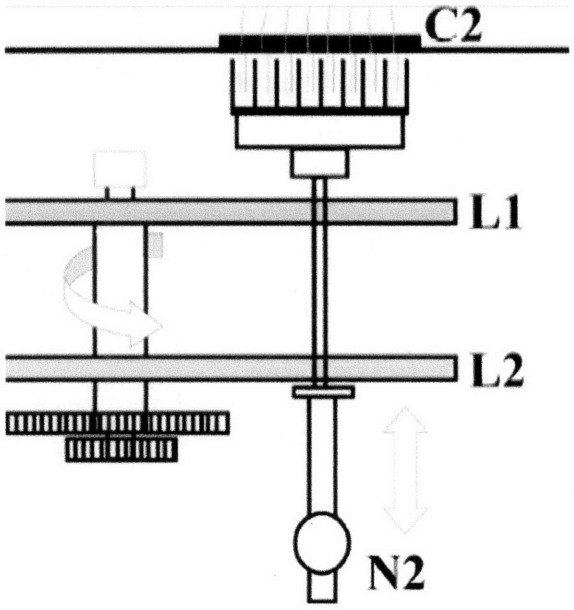
During operation, the fraction collection module positions the agarose tray to align its first row of wells under the 1 x 24 capillary array at the fraction collection end. The agarose tray alignment subsystem's video camera captures images in the entrance area where the agarose tray moves toward the fraction collection end of the capillary array. Thus, the function of this video camera is to monitor the alignment between the first row of the agarose tray and the capillary array's fraction collection end [4].

Subsequently, the image from the video camera is displayed on a second personal computer and is processed by an alignment software module written in LabView to calculate the angular offset in parallelism between the rows of the agarose tray and the fraction collection ends of the capillary array. If any irregularity is spotted, it can be corrected immediately by adjusting the rotational stage beneath the Delrin tray. Initially, the first row of the agarose tray plays an important part: as soon as the first row of the agarose tray parallel to the capillary fraction collection ends is verified, the agarose tray is

translated in the x-direction so that each row of wells is presented sequentially to the capillary array. The dwell time for the capillary in each row of wells ranges from 30 seconds to a minute. Additionally, a programmed delay is created before the translation from the first to the second row for the purpose of allowing the fastest migrating portions of the injected sample to arrive at the end of the capillary [4].

5.1.6. Sample Delivery and Injection

Adapted from the SCE2410 DNA sequencer, the HTMS design for both sample delivery and injection is slightly modified. In this modified design, a pair of programmable carousels (L1, L2), connected with a pneumatic stage (N2), provide the integrated automation for sample delivery and injection [4]. See below:



(Part of **Figure 16**)

5.1.7. Automation Control and Software

Two personal computers provide automation control for the HTMS: these two PCs can communicate with each other via an Ethernet connection. The first PC from the SCE2410 DNA sequencer, using its original software, handles the regular instrumental operations, such as sample delivery, capillary array filling and washing, electrophoresis, and data acquisition. The second PC, using LabVIEW software, handles all requests from the first PC, and controlls all the add-on modules to run automated CDCE experiments, such as high pressure chamber (HPC) pneumatics, the solid-state heaters, the fraction collection, and the agarose tray alignment subsystem [4].

5.2. PCR

This PCR section reported by Q. Li *et al.* as follows [4]: Two target sequences were selected from the human thiopurine methyltransferase (TPMT) gene and one from the human cytotoxic T lymphocyte associated-4 (CTLA-4) gene. The first two fragments, TPMT2-1B and TPMT3-1B, derived from exon 4 and exon 5 of the TPMT gene [4, 70]. The third fragment was CTLA-4-E1Nsa, derived from exon 1 of the CTLA-4 gene. Target sequences were selected by the following software: WinMelt 2.0 (Medprobe, Oslo, Norway) and Primer Premier 5 (Premier Biosoft International, Palo Alto, CA) software [4].

The PCR product sizes for TPMT2-1B, TPMT3-1B, and CTLA-4 were 176 bp, 173 bp (172 bp for mutant), and 186 bp, respectively. Table 1 below displaying the PCR primers used to amplify the TMPT2-1B, TMPT3-1B, and CTLA-4-E1Nsa fragments [4].

Table 1: PCR primers used in amplification of TMPT2-1B, TMPT3-1B, and CTLA-4-E1Nsa

Fragment	Primers	Fragment Size (bp)
TMPT2-1B	5'-GAG CCC CCG CCC CGC CCG CCG ACC CCC GCC CCG CCC GCC GAG AAA CCT TTT TTT TTT TCC TC-3' and 5'F-ATA CCA TTT CAT CTC AAC CG-3'	176 bp
TMPT3-1B	5'-GCC GCC TGC AGC CCG CGC CCC CCG TGC CCC CGC CCC GCC GCC GGC CCG GGC GCC CTT CTG AGT AAG AAA GAT TCT G3' and 5'-F- GTG TAA ATG TAT GAT TTT ATG CAG GTT TGC-3'	173 bp
CTLA-4-E1Nsa	5'-GCC GCC TGC AGC CCG CGC CCC CCG TGC CCC CGC CCC GCC GCC GGC CCG GGC GCC GCA GAA GAC AGG GAT GAA GA-3' and 5'-F- ACC TGA ACA CCG CTC CCA TA-3'	186 bp

Reported by Q. Li *et al.*as: Genomic DNA was derived from a pooled blood sample of 2,000 juveniles [4, 33, 60]. Genomic DNA at 0.25 to 0.50 ng was used as the starting template for high-fidelity PCR in 20μL reaction volume in 96-well thermocyclers (MWG, High Point, NC). The PCR mixtures contained 20mM Tris-HCI (pH 8.0), 2mM $MgCI_2$, 10mM KCI, 6mM $(NH4)_2SO_4$, 0.1% Triton X-100, and 0.1 mg/mL BSA, 0.1 mM dNTPs, 0.2 μM of each primer and 0.1 U/ μL HotStart Turbo Pfu (Stratagene, La Jolla, CA) [4].

The PCR reaction condition reported exactly as: "denaturation 95^0C for 2 minutes; then 35-40 cycles of 30 seconds at 95^0C, 40 seconds at 50-55^0C, and 40 seconds at 72^0C; 72^0C for 2 minutes; and 45^0C for 15 minutes. For CTLA-4-E1Nsa, heteroduplexes were

formed by boiling the PCR product solutions for 5 minutes and reannealing at 65^0C for 1 hour. For TPMT2-1B and TPMT3-1B, heteroduplexes were reconstructed by mixing the PCR product of the artificial and wild-type mutants at a specific ratio followed by boiling for 5 minutes and reannealing at 65^0C for 1 hour. Finally, the resulting samples are diluted with water prior to electrokinetic injection into the capillaries" [4].

Chapter 6

Results and Discussion

Since its introduction in 1994, CDCE has been acknowledged as a method of mutation detection with high sensitivity. Utilizing the theory of differential melting of DNA, this multicapillary HTMS instrument has demonstrated its ability to meet the strict CDCE requirements for high-sensitivity detection and low-frequency measurement of rare variants in large population studies [20]. In the following section, this thesis will discuss HTMS experiment results in the areas of: (1) detection sensitivity and dynamic range, (2) CDCE temperature optimization and separation reproducibility, (3) fraction collection and mutant enrichment, and (4) HTMS process with flexible strategies for aiming at small or large pools of population study [4].

6.1 Optical Detection Sensitivity, Dynamic Range, and Crosstalk

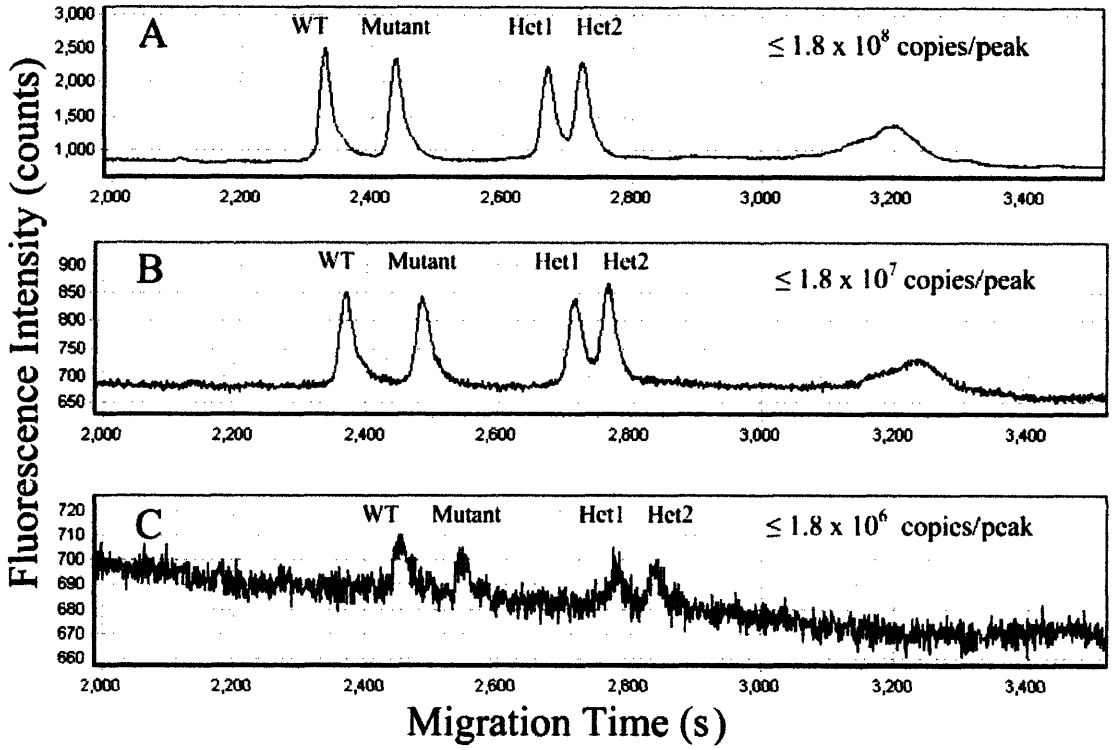
As discussed in Chapter 5, the modifications incorporated in HTMS array layout, imaging lens design, and wavelength selection have helped to improve its optical sensitivity and dynamic range, and reduced crosstalk. Pertinent performances on HTMS carried out reported by *Q. Li et al.* as follows:

(1) Signal-to-noise ratio (S/N) by flow fluorescein experiments: First, the S/N was selected for each capillary by using its peak-to-peak base-line noise. Then the average S/N

was acquired for all 24 capillaries. The results indicated a detection limit of about 1 x 10^{-12} M fluorescein across the array. The dynamic range of the HTMS system was $10^{3.5}$ [4].

(2) Next, the test on optical detection sensitivity measured by CDCE: optical detection sensitivity was measured by CDCE using fluorescein labeled DNA sample TPMT2-1B. TPMT3-1B was injected at three concentrations of 2.4 x 10^8, 2.4 x 10^7, 2.4 x 10^6 copies/μL each from 5μL sample. Then using the calibration method, the amount of DNA injected was around 7.2 x 10^8, 7.2 x 10^7, and 7.2 x 10^6 copies each. The electropherograms obtained by CDCE for each sample concentration indicated wild-type, mutant, and two heteroduplexes with very similar peak sizes. The number of DNA molecules per peak was estimated to be 1.8 x 10^6 as shown in **Figure 14** below [4]:

Figure 14:



Optical detection sensitivity measured by CDCE *(with permission)*

The above figure indicated the estimation of detection limit in number of DNA molecules for the HTMS. The experimental results indicated a detection limit of 2×10^6 DNA molecules per peak in the HTMS. However, since the labeling efficiency for a primer is generally lesser than 100%, the detection limit is believed to be lower than 2×10^6 copies [4].

Test on the crosstalk was also carried out as: injecting concentrated and diluted TPMT2-1B samples into alternating capillaries to determine the level of crosstalk. Only results for half of the array were shown. Every odd capillary which was injected with the concentrated sample showed a large fluorescence peak with intensities from 15,000 to

32,000 counts between migration time 1100 and 1200 s. However, the even capillaries did not show any measurable peaks within the same migration time window. This indicated that no crosstalk higher than 0.06% was detected in the system. The crosstalk below such a level could keep the precise focusing of the imaging lens and alignment of the capillary cartridge [4].

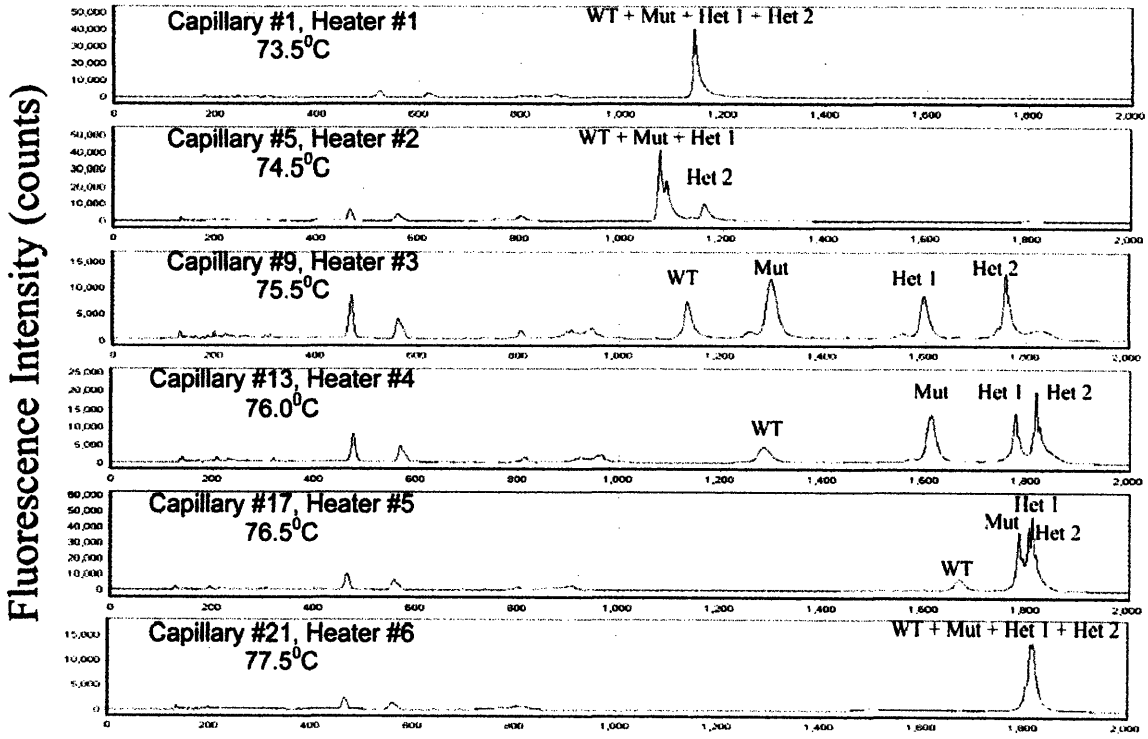
6. 2. CDCE Temperature Optimization and Separation Reproducibility

As discussed in Chapter 3, target sequences subject to CDCE analysis have high and low domains, which in turn will affect DNA mobility [7, 10]. In order to maximize the differences in mobility for DNA molecules and keep the separation reproducibility, accurate and precise temperature control of the sample is implemented during the CDCE process. In principle, any tiny temperature change will affect mobility shift. Therefore, it is very important to maintain accurate and precise temperature control during the CDCE process.

In the HTMS system, six independently controlled solid-state thermal heaters for the precision thermostat array for CDCE temperature control system were utilized to optimize the CDCE temperature for a target sequence in a single run. In order to achieve the optimization of the CDCE temperature, the melting temperature (T_m) of a target sequence was first calculated by using WinMelt 2.0 [60]. Then, the six heaters in the thermostat array were set at different temperatures within a narrow range, typically several degrees, centering on the calculated T_m. The next step was to inject the same DNA sample into at least one of the four capillaries in each heater, which led to a simultaneous

separation of the sample at six different temperatures (4 x 6 = 24). Subsequently, the optimum CDCE temperature was identified by the CDCE electropherogram indicating the maximum resolution among the four DNA species, including wild-type, mutant, and heteroduplexes [4]. **Figure 15** shows the CDCE temperature optimization for CTLA-4-E1Nsa was 75.5^0 C [4].

Figure 15:



Temperature Optimization of target sequence CTLA-4-E1Nsa

Temperature optimization of target sequence CTLA-4-E1Nsa. The CTLA-4-E1Nsa sample was injected into capillaries 1, 5, 9, 13, 17, and 21 in heaters 1, 2, 3, 4, 5, and 6, respectively. Starting from the top panel representing the lowest temperature, and moving toward lower panels representing increasing temperature, the single DNA peak starts to resolve gradually into four peaks, then coalesces back into one peak again at the highest temperature. The results show that the optimum CDCE temperature for CTLA-4-E1Nsa is 75.5^0 C.
(with permission)

For the purpose to maximize the differences in mobility for the DNA molecules and to maintain CDCE separation reproducibility, it is imperative to implement accurate and precise temperature control of the sample over a significant length of the separation column during the CDCE process, as described above. Records from Q. Li *et al.* indicated that a temperature change as small as 0.05^0C was sufficient to induce an observable mobility shift. Furthermore, records also indicated that minute oscillations as small as ± 0.1^0C in a capillary's temperature resulted in significant degradation in the number of theoretical plates. Consequently, it was crucial that accuracy and precision better than ± 0.05^0C be maintained to guarantee reproducibility in the CDCE process if isothermal temperature control was employed [4].

6. 3. Fraction Collection and Mutant Enrichment

Early in 1997, MIT's Thilly group developed a method to separate mutant from nonmutant DNA sequences on the basis of their melting temperatures and then increasing the number of copies by hifi DNA amplification that results in point mutations in biological samples at fractions at 10^{-6} or above [7]. Usually, after amplification by hifiPCR using fluorescein-labelled primers, mutants are further enriched by two more rounds of CDCE coupled with hifiPCR. At that time, individual mutants, observed as distinct peaks on CDCE, are then isolated and sequenced [7, 10]. Since this approach described herein is applicable to general DNA sequences suitable for CDCE analysis, Q. Li's fraction collection system mirrored a similar process, except his approach was taken to

calibrate the fraction location on the agarose plate versus the peak migration times displayed on electropherograms [4].

Q. Li *et al.* reported to run a typical HTMS experiment as follows, using TPMT2-1B for explanation:

(1) For initial calibration, TPMT2-1B sample mixtures were injected in alternative capillaries. (2) Separated DNA fragments in each capillary were electroeluted into 30 wells with approximately 30 seconds per well dwelling time. (3) Solutions in all the 1536 wells (48 x 32) were transferred into four 384-well plates using a Hydra liquid handler. (4) The 720 fractions from the 24 capillaries (24 x 30 = 720) were transferred to 96-well plates for PCR amplification. (5) The amplified products of selected fractions from capillaries injected with blank buffer were run on the HTMS to verify that no cross contamination occurred. (6) For the fractions eluted from the capillaries, the amplified products were run in the HTMS to determine the wells containing the wild-type and mutant fractions, based on CDCE peak profile and signal strength. (7) The results were utilized to calibrate the fraction well position on the agarose plate predicted from the migration time of a peak. (8) Finally, after the separation run was completed, the desired fractions were selected using sample profiles recorded during the separation experiment [4].

For future improvements in migration time reproducibility, Li *et al.* suggested using coated capillaries and accurate high temperature control for the capillary portions outside the thermostat heaters to reduce wells required to have undergone PCR amplification to identify the fractions of interest [4].

In this HTMS model, the use of the automation of the replaceable sieving matrix and agarose-based collection plate not only helped to minimize evaporation of the fraction during the run, but also made it a high-throughput mutation detection instrument [4].

6. 4. The HTMS Process and Strategies

This CDCE-based HTMS process includes the following steps in sequence: hifiPCR, CDCE, fraction collection, hifiPCR, and CDCE. From experiments run, results derived as follows: optical dynamic range is $10^{3.5}$, optical detection limit is 2 x 10^6 DNA molecules, and its fraction collection enrichment is 10-fold, and the resulting theoretical HTMS process detection limit is $10^{-4.5}$ [4].

Utilizing this HTMS process, two different strategies for mutation discovery in pooled samples can be used for population studies as reported by Q. Li *et al.*:

The first strategy is related to multiple small pools (i.e., 96 individuals each), which can be created from each studied population. Each of this type of small pool can be directly analyzed by hifiPCR and CDCE in the HTMS, permitting for up to 24 pools of 96 individuals to be simultaneously analyzed in each CDCE run [60]. For mutations with low-detection frequency in this group, mutant identification can also be achieved through the HTMS process [4].

The second strategy is that each study population can be scrutinized as a single large pool [11]. Since CDCE allows high-resolution separation of single-base variations occurring in an ~100 bp isomelting DNA sequence based on their differential melting temperature, by coupling CDCE for highly efficient enrichment of mutants with hifiPCR,

the sample can be separated using HTMS. Moreover, the heteroduplex fraction is collected to enrich the mutant using the automated fraction collection. The process for the PCR, separation, and collection can be repeated once or twice until mutants become visible on the HTMS [7, 10, 4].

In sum, these two strategies can be utilized as a powerful and cost-effective tool for population studying in multiple small pools or single large pools [4].

Chapter 7

Conclusions

Within the past decade, CDCE has grown from a laboratory research project to an established separation tool based on a cooperative melting equilibrium principle that is used to detect mutations in 100 bp sequences with a measurable frequency as low as 10^{-6}. In other words, CDCE is a powerful tool for detecting low-frequency mutations in human cells and tissues in single capillary instruments. However, it cannot be said that using CDCE is a practical tool in mutation detection in large population studies, due to the instrument's lack of flexibility, automation, and other throughput requirements. In addition, lack of cost effectiveness for large size studies is another practical consideration.

Since in principle CDCE can be adapted to any commercially available DNA sequencing instrument in either a single-capillary or multicapillary system, with or without structural modification, this thesis has selected an integrated automated multicapillary instrument with collection of mutant fractions by using CDCE to meet the stringent requirements for detecting low-frequency mutations in pooled samples from large populations. A substantially modified SCE2410 24-capillary DNA Sequencer, this prototype instrument, named the HTMS Model (High-throughput Mutaitonal Spectrometer) by Q. Li *et al.,* has been identified as the instrument that best meets these requirements.

The overall design of this thesis was two folds: First is on DNA and CDCE separation theory and technology, two is on HTMS -- to analyze the HTMS as a CDCE-based mutational spectrometer, with a focus on illuminating important aspects of the design and the end results of using the equipment. From a design angle, this study attempted to render a critical assessment on the HTMS' substantial modifications to its capillary cartridge, its modules of temperature control, optical detection, automated matrix replacement and fraction collection and analyze them in terms of strict CDCE requirements for each category. From the end-results angle, this study analyzed experiments by Q. Li *et al.* on the major integrated HTMS modules that offer CDCE advantages, such as sensibility, low-frequency detection, reproducibility, throughput, and cost-effectiveness as summarized below.

First, the highly efficient optical design has rendered an extremely robust detection system to the HTMS, which has achieved desirable optical detection sensitivity (about 1 x 10^{-12} M fluorescein across the array), dynamic range (of $10^{3.5}$), and reduced levels of crosstalk (below 0.02% for the 20 capillaries in the center of the array, and 0.03-0.05% for the 2 capillaries on each side).

Second, the precision thermostate array for CDCE in this instrument has been achieved with the use of six solid-state heaters, each individually controlled to maintain an accurate temperature of ±0.013°C in their respective capillaries, thus maintaining reproducibility in the CDCE process.

Thirdly, to achieve cost effectiveness and throughput, large pool sample analysis requires the aforementioned requirements as well. The CDCE-based HTMS provides two

different strategies -- multiple small pools and single large pool -- for mutation discovery in pooled samples. In the Li-Sucholelki *et al.* 2005 study of detection of rare variants in pools of genomic DNA from large populations using mutational spectrometry, this CDCE-based approach was a powerful, cost-effective tool for the study of both multiple small pools and single large pools [20].

In sum, the discussion above supports the significance of CDCE as a separation tool for various genetic study applications. In accordance with Morgenthaler and Thilly's pangenomic study on discovery genes that carrying multi-allelic or mono-allelic risk for common diseases, the conclusion of this study is that any expansion or development of similar devices that allow automated parallel separation and collection of all fractions and fraction analysis will make the detection of the 100 most common diseases in populations of one million people or more a feasible reality from both technological and economic vantage points [71].

REFERENCES

1. Cotton, R.G.H. Current methods of mutation detection. *Mutation Research*, **285**: 125-144 (1993).

2. Lander, E.S. *et al.* Initial sequencing and analysis of the human genome. *Nature* **409** (6822): 860-921 (February 21, 2001).

3. Tomita-Mitchell, A., Muniappan, B.P., Herrero-Jimenez, P., Zarbl, H., and Thilly, W.G. Single nucleotide polymorphism spectra in newborns and centenarians: identification of genes coding for rise of mortal disease. *Gene* **223**: 381-391 (1998).

4. Li, Qingbo, Deka, C., Glassner, B.J., Arnold, K., Li-Sucholelki, X., Tomita-Michell, A., Thilly, W.G., and B.L. Karger. Design of an automated multicapillary instrument with fraction collection for DNA mutation discovery by constant denaturant capillary electrophoresis (CDCE). *J. Sep. Sci.* **28:** 1375-1389 (2005).

5. Bjorheim, J. and Ekstrom, P.O. Review of denaturant capillary electrophoresis in DNA variation analysis. *Electrophoresis* **26**: 2520-2530 (2005).

6. Khrapko, K., Hanekamp, J.S., Thilly, W.G., Belenkii, A., Foret, F., and Karger, B.L. Constant denaturant capillary electrophoresis (CDCE): A high resolution approach to mutational analysis. *ucleic Acid Research* **22 (3):** 364-369 (1994).

7. Khrapko, K., Coller, H., André, P., Li, Xiao-Cheng, Foret, F., Belenky, A., Karger, B.L. and Thilly, W.G. Mutational spectrometry without phenotypic selection: human mitochondrial DNA. *Nucleic Acid Research* **25 (4):** 685-693 (1997).

8. Kim, A. S., Li-Sucholeiki, X., and Thilly, W.G. Applications of constant denaturant capillary electrophoresis and complementary procedures: Measurement of point mutational spectra. In Mitchelson, K.R., and Cheng, J., eds. *Capillary Electrophoresis of Nucleic Acids*. Totowa, N.J.: Humana Press, Volume **2**: 175-189 (2001).

9. Khrapko, K., Coller, H.A., Li-Sucholeiki, X.C., Andre, P.C., and Thilly, W.G. High Resolution Analysis of Point Mutations by Constant Denaturant Capillary Electrophoresis (CDCE). In Mitchelson, K.R., and Cheng, J., eds. *Capillary*

Electrophoresis of Nucleic Acids. Totowa, N.J.: Humana Press, Volume **2**: 57-72 (2001).

10. Li-Sucholelki, X. and W. G. Thilly. A sensitive scanning technology for low frequency nuclear point mutations in human genomic DNA. *Nucleic Acids Research* **28 (9):** e44 (2000).

11. Li-Sucholelki, X., Khrapko, K., André, P.C., Marcellno, L.A., Karger, B.L., and Thilly, W.G. Applications of constant denaturant capillary electrophoresis/high-fidelity polymerase chain reaction to human genetic analysis. *Electrophoresis*, **20**: 1224-1232 (1999).

12. Khrapko, K., Coller, H., and Thilly, W.G. Efficiency of separation of DNA capillary electrophoresis is controlled by the kinetics of DNA melting equilibrium. *Electrophoresis* **17:** 1867-1874 (1996).

13. Mitchelson, K.R. and Cheng, J., eds. *Capillary Electrophoresis of Nucleic Acids: Introduction to the Capillary Electrophoresis of Nucleic Acids*, Vols. I and II. Totowa, New Jersey: Humana Press, 2001.

14. Righetti, P.G., Gelfi, C., D'Acunto, M.R. Recent progress in DNA analysis by capillary electrophoresis. *Electrophoresis* **23**: 1361-1374 (2002).

15. Ekstrom, P.O., Børresen-Dale, A.L., Qvist, H., Giercksky, K.E., and Thilly, W.G. Detection of low-frequency mutation in exon 8 of the TP53 gene by constant denaturant capillary electrophoresis (CDCE). *Biotechniques* **27**: 128-134 (1999).

16. Fischer, S.G. and L.S. Lerman. Separation of random fragments of DNA according to properties of their sequences. *Proc. National Academy of Sciences,* **77 (8):** 4420-4424 (1980).

17. Fischer, S.G. and L.S. Lerman. DNA fragments differing by single base-pair substitutions are separated in gradient gels: correspondence with melting theory. *Proc. National Academy of Sciences* **80 (6)**: 1579-1583 (1983).

18. Li-Sucholelki, X. and W.G. Thilly. Use of wide-bore capillaries in constant denaturant capillary electrophoresis. *Electrophoresis* **17**: 1884-1889 (1996).

19. Li-Sucholelki, X., Hu, G., Perls, T., Tomita-Mitchell, A., and Thilly, W.G. Scanning the beta-globin gene for mutations in large populations by denaturing capillary and gel electrophoresis. *Electrophoresis* **26**: 2531-2538 (2005).

20. Li-Sucholeiki, X., Tomita-Mitchell, A., Arnold, K., Glassner, B.J., Thompson, T.,

Murthy, J.V., Berk, L., Lange, C., Leong-Morgenthaler, P.M., MacDougall, D., Munro, J., Cannon, D., Mistry, T., Miller, A., Deka, C., Karger, B.L., Gillespie, K.M., Ekstrom, P.O., Todd, J.A., and Thilly, W.G. Detection and frequency estimation of rare variants in pools of genomic DNA from large populations using mutational spectrometry. *Mutation Research* **570**: 267-280 (2005).

21. Muniappan, Brindha P. and William G. Thilly. Application of constant denataurant capillary electrophoresis (CDCE) to mutation detection in humans. *Genetic Analysis* **14**: 221-227 (1999).

22. Hovig, E., Smith-Sorensen, B., Brogger, A., and Borresen, A. L. Constant denaturant gel electrophoresis, a modification of denaturing gradient gel electrophoresis in mutation detection. *Mutation Research* **262**: 63-71 (1991).

23. Cohen, A.S., Najarian, D.R., Paulus, A., Guttman, A., Smith, J.A., and Karger, B.L. Rapid separation and purification of oligonucleotides by high-performance capillary gel electrophoresis. *Proc. National Academy of Sciences* **85 (24)**: 9660-9663 (1988).

24. Khrapko, K., Coller, H.A., Hanekamp, J.S., and Thilly, W.G. Identification of point mutations in mixtures by capillary electrophoresis hybridization. *Nucleic Acids Research* **26 (4):** 5738-5740 (1998).

25. Kumar, R., Hanekamp, J.S., Louhelainen, J., Burvall, K., Onfelt, A., Hemminki, K., and Thilly, W.G. Separation of transforming amino acid-substituting mutations in codons 12, 13, and 61 of the N-*ras* gene by constant denaturant capillary electrophoresis (CDCE). *Carcinogenesis* **16 (11):** 2667-2673 (1995).

26. From http://rodium.no/sfg.

27. Bjorheim, J., Gaudernack, G., and Ekstrom, P.O. Melting gel techniques in single nucleotide polymorphism and mutation detection: From theory to automation. *J. Sep. Sci.* **25**: 637-647 (2002).

28. Ekstrom, P.O., Wasserkort, R., Minarik, M., Foret, F., and Thilly, W.G. Two-point fluorescence detection and automated fraction collection applied to constant denaturant capillary electrophoresis. *Biotechniques 29***:** 582-589 (2000).

29. Bjorheim, J., Lystad, S., Lindblom, A., Kressner, U., Westriing, S., Wahlberg, S., Lindmark, G., Gaudernack, G;, Ekstrom, P.O., Roe, J., Thilly, W.G., and Borresen-Dale, A.L. Mutation analyses of *KRAS* exon 1 comparing three different techniques: temporal temperature gradient electrophoresis, constant denaturant capillary electrophoresis and allele specific polymerase chain reaction. *Mutation Research* **403**: 103-112 (1998).

30. Ekstrom, P.O. and Bjorheim. J. Evaluation of sieving matrices used to separate alleles by cycling temperature capillary electrophoresis. *Electrophoresis* **27**: 1878-1885 (2007).

31. Coller, Hilary A., Khrapko, K., Bodyak, N.D., Nekhaeva, E., Herrero-Jimenez, P., and Thilly, W.G.. High frequency of homoplasmic mitochondrial DNA mutations in human tumors can be explained without selection. *Nature Genetics* **28**: 147-150 (2001).

32. Keohavong, Phouthone and William G. Thilly. Fidelity of DNA polymerases in DNA amplification. *Proc. National Academy of Sciences* **86 (23):** 9253-9257 (1989).

33. Ekstrom, P.B., Bjorheim, J., Gaudernack, G., and Giercksky, K.E. Population screening of single-nucleotide polymorphisms exemplified by analysis of 8000 alleles. *J. Biomolecular Screening* **7(6):** 501-506 (2002).

34. Bjorheim, J., Ekstrom, P.O., Fossberg, E., Børresen-Dale, A.L., and Gaudernack, G. Automated constant denaturant capillary electrophoresis applied for detection of *KRAS* exon 1 mutations. *BioTechniques* **30 (5)**: 972-975 (2001).

35. Bjorheim, J., Minarik, M., Gaudernack, G., and Ekstrom, P.O. Mutation detection in *KRAS* exon 1 by constant denaturant capillary electrophoresis in 96 parallel capillaries. *Analytical Biochemistry* **304**: 200-205 (2002).

36. Guldberg, P. and Guttler, F. A simple method for identification of point mutations using denaturing gradient gel electrophoresis. *Nucleic Acid Research* **21 (9):** 2261-2262 (1993).

37 Minarik, M., Minarikova, L., Bjorheim, J., Ekstrom, P.O. Cycling gradient capillary electrophoresis: A low-cost tool for high-throughput analysis of genetic variations. *Electrophoresis* **24**: 1716-1722 (2003).

38. Kristensen, A.T., Bjorheim, J., Minarik, M., Giercksky, K.E., and Ekstrom, P.O. Detection of mutations in exon 8 of *TP53* by temperature gradient 96-capillary array electrophoresis. *BioTechniques* **33 (3):** 650-653 (2002).

39. Bjorheim, J., Minarik, M., Gaudernack, G., and Ekstrom, P.O. Evaluation of denaturing conditions in analysis of DNA variants applied to multi-capillary electrophoresis instruments. *J. Sep. Sci.* **26**: 1163-1168 (2003).

40. Bjorheim, J. *Automated Denaturant Capillary Electrophoresis in DNA Variation Analysis*. Ph.D. Thesis. Oslo, Norway, 2003.

41. Schwartz, H. and Guttman, A. *Separation of DNA by Capillary Electrophoresis* (Beckman Primer 607397). Fullerton, CA, 1995. Also available: www. Beckman.com/literature/Bioresearach

42. Altria, K.D., ed. *Capillary Electrophoresis Guidebook: Principles, Operation, and Applications*. Totowa, New Jersey: Humana Press, 1996.

43. Bjorheim, J., Gaudernack, G., and Ekstrom, P.O. Mutation analysis of *TP53* exons 5-8 by automated constant denaturant capillary electrophoresis. *Tumor Biology* **22**:323-327 (2001).

44. Bjorheim, J., Gaudernack, G., Giercksky, K.E., and Ekstrom, P.O. Direct identification of all oncogenic mutants in *KRAS* exon 1 by cycling temperature capillary electrophoresis. *Electrophoresis* **24**: 63-69 (2003).

45. Poland, D. Recursion relation generation of probability profiles for specific-sequence macromolecules with long-range correlations. *Biopolymers* 13: 1859-1871 (1974).

46. Fixman, M. and Preire, J.J. Theory of DNA melting curves. *Biopolymers* **16**: 2693-2704 (1977).

47. Steger, G. Thermal denaturation of double-stranded nucleic acids: prediction of temperatures critical for gradient gel electrophoresis and polymerase chain reaction. *Nucleic Acids Research* **22** (12): 2760-2768 (1994).

48. Børresen, A.L., Hovig, E., Smith-Sorensen, B., Malkin, D., Lystad, S., Andersen, T.I., Nesland, J.M., Isselbacher, Friend, S.H. Constant denaturant gel electrophoresis as a rapid screening technique for p53 mutations. *Proc. National Academy of Sciences* **88 (19)**: 8405-8409 (1991).

49. Bjorheim, J., Abrahamsen, T.W., Kristensen, A.T., Gaudernack, G., and Ekstrom, P.O. Approach to analysis of single nucleotide polymorphisms by automated constant denaturant capillary electrophoresis. *Mutation Research* **526**: 75-83 (2003).

50. *Machinery's Handbook* (26th edition). New York: Industrial Press Inc. 2000.

51. Crane, Bryan L., Hogan, N.C., Sudo, H., Thilly, W.G., and Hunter, I. W. Realtime PCR Measurement by Fluorescence anisotrophy. Analytical Chemistry 77 (16) 5129-5134 (2005).

52. Myers, R.M., Fischer, S.G., Lerman, L.S., and Maniatis, T. Nearly all single base substitutions in DNA fragments joined to a GC-clamp can be detected by denaturing gradient gel electrophoresis. *Nucleic Acids Research* **13 (9):** 3131-3145 (1985).

53. Sheffield, V.C., Cox, D.R., Lerman, L.S., and Myer, R.M. Attachment of a 40-base-pair G + C-rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes. *Proc. National Academy of Sciences* **86:** 232-236 (1989).

54. *http://www.appliedbiosystems.com*
http://www.apbiotech.com
http://www.beckmancoulter.com
http://www.megabace.net/megabace/
http://www.apbiotech.com/MegaBACE
http://www.megabace.net/megabace/
http://www.phrap.org
http://www.spectrumedix.com (SpectrumMedix)
http://www.watrex.com

55. Condie A., Eeles, R., Borresen A.L., Coles, C., Cooper, C., Prosser, J. Detection of point mutations in the p53 gene: comparison of single-strand conformation polymorphism, constant denaturant gel electrophoresis, and hydroxylamine and osmium tetroxide techniques. *Hum Mutation* **2**(1): 58-66 (1993).

56. Cohen, A.S.and Karger, B.L. Highh performance capillary electrophoresis using open tubes and gels. *Chromatography* **397:** 409-417 (1987).

57. Kim, A., Khrapko, K., and Thilly, W.G. Fidelity and mutational spectrum of *Pfu* DNA polymerase on a human mitochondrial DNA sequence. *Genome Research* **7 (8):** 843-852 (1997).

58. Kim, A. S., Holmquist, G.P. and Thilly, W.G. High-efficiency DNA ligation for clamp attachment without polymerase chain reaction. *Anal. Biochem.* **310**: 179-185 (2002).

59. Kim, A. S. and Thilly, W.G. Ligation of high-melting-temperature 'clamp' sequence extends the scanning range of rare point mutational analysis by constant denaturant capillary electrophoresis (CDCE) to most of the human genome. *Nucleic Acids Research* **31**, No. 16 e97 (2003).

60. Xue, Ming-Zhan, Bonny, O., Morgenthaler, S., Bochud, M., Mooser, V., Thilly, W.G., Schild, L., and Leong-Morgenthaler, P.M. Use of constant denaturant

capillary electrophoresis of pooled blood samples to identify single-nucleotide polymorphisms in the genes (*scnn l a* and *scnn l b*) encoding the α and ß subunits of the epithelial sodium channel. *Clinical Chemistry* **48 (5)**: 718-728 (2002).

61. Li, Qingbo, Liu, Z., Monroe, H., Culiat, C.T. Integrated platform for detection of DNA sequence variants using capillary array electrophoresis. *Electrophoresis* **23**: 1499-1511 (2002). Also see http://www.spectrumedixcom.

62. "Multichannel microscale system for high throughput preparative separation with comprehensive collection and analysis." U.S. Patent No: US 6,660,149 B1 (Dec. 9, 2003). Inventors: Barry L. Karger, L.Kotler, F. Foret, M. Minarik and K. Kleparnik

63. Muller, O., Foret, F., Karger, B, L. Design of a high-precision fraction collector for capillary electrophoresis. Anal. Chem. 67:2974-2980 (1995).

64. Magnusdottir, S., Heller, C., Sergot, P., and Viovy, J. "Collection of capillary electrophoresis fractions on a moving membrane." In Mitchelson, K.R. and Cheng J., eds. *Capillary Electrophoresis of Nucleic Acids: Introduction to the Capillary Electrophoresis of Nucleic Acids*, Vol. I. Totowa, New Jersey: Humana Press, 2001.

65. Minarik, M., Foret, F., Karger, B.L. Fraction collection in micropreparative capillary zone electrophoresis and capillary isoelectric focusing. *Electrophoresis* **21**: 247-254 (2000).

66. Ulrich, K.T. and Eppinger, S.D. *Product Design and Development* (third edition). New York: The McGraw-Hill Companies, 2004.

67. Irie, T., Oshida, T., Hasegawa, H., Matsuoka, Y., Li, T., Oya, Y., Tanaka, T., Tsujimoto, G., and Kambara, H. Automated DNA fragment collection by capillary array gel electrophoresis in search of differentially expressed genes. *Electrophoresis* **21**: 367-374 (2000).

68. Minarik, M., Kleparnik, K., Gilar, Martin, Foret, F., Miller, A.W., Sosic, Z., Karger, B.L. Design of a fraction collector for capillary array electrophoresis. *Electrophoresis* **23**: 35-42 (2002).

69. Berka, J., Ruiz-Martinez, M.C., Hammond, R., Minarik, M., Foret, F., Sosic, Z., Kleparnik, K., and Karger, B.L. Application of high-resolution capillary array electrophoresis with automated fraction collection for GeneCalling analysis of the yeast genomic DNA. *Electrophoresis* **24**: 639-647 (2003).

70. http://www/mcbo.nih.gov.

71. Morgenthaler, Stephan and W.G. Thilly. A strategy to discover genes that carry multi-allelic or mono-allelic risk for common diseases: A cohort allelic sums test (CAST). *Mutation Research* **615**: 28-56 (2007).